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THREE-DIMENSIONAL ENDOTHELIAL SPHEROID-BASED INVESTIGATION OF PRESSURE-SENSITIVE SPROUT FORMATION

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THREE-DIMENSIONAL ENDOTHELIAL SPHEROID-BASED INVESTIGATION OF PRESSURE-SENSITIVE SPROUT FORMATION

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biomedical Engineering
in the College of Engineering
at the University of Kentucky

By

Min Song

Director: Dr. Hainsworth Shin, Professor of Biomedical Engineering

Lexington, Kentucky

Abstract

THREE-DIMENSIONAL ENDOTHELIAL SPHEROID-BASED INVESTIGATION OF PRESSURE-SENSITIVE SPROUT FORMATION

This study explored hydrostatic pressure as a mechanobiological parameter to control in vitro endothelial cell tubulogenesis in 3-D hydrogels as a model microvascular tissue engineering approach. For this purpose, the present investigation used an endothelial spheroid model, which we believe is an adaptable microvascularization strategy for many tissue engineering construct designs. We also aimed to identify the operating magnitudes and exposure times for hydrostatic pressure-sensitive sprout formation as well as verify the involvement of VEGFR-3 signaling. For this purpose, we used a custom-designed pressure system and a 3-D endothelial cell spheroid model of sprouting tubulogenesis. We report that an exposure time of 3 days is the minimum duration required to increase endothelial sprout formation in response to 20 mmHg. Notably, exposure to 5 mmHg for 3 days was inhibitory for endothelial spheroid lengths without affecting sprout numbers. Moreover, endothelial spheroids exposed to 40 mmHg also inhibited sprouting activity by reducing sprout numbers without affecting sprout lengths. Finally, blockade of VEGFR-3 signaling abolished the effects of the 20-mmHg stimuli on sprout formation. Based on these results, VEGFR-3 dependent endothelial sprouting appears to exhibit a complex pressure dependence that one may exploit to control microvessel formation.

KEYWORDS: Mechanotransduction, Angiogenesis, Lymphangiogenesis, Tissue Engineering, Vascular Endothelial Growth Factor, Microcirculation

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Table of Contents

List of Figures

List of Tables

1. Introduction

According to the U.S. Department of Health & Human Services, more than 120,000 patients are on waiting lists for transplantation surgery in the end of 2015 (U.S. Department of Health & Human Services, 2016). Despite this, only around 31,000 transplants are performed in 2015 with an average of 22 people dying each day while waiting for a transplant (U.S. Department of Health & Human Services, 2016). To amplify this problem, the need for transplants continues to increase. Because of this high demand for donor organs coupled with their limited availability, there has been much efforts dedicated to designing strategies to tissue engineer organs as suitable substitutes to improve or replace lost tissues. Even though people have been putting tremendous efforts in tissue engineering, this organ crisis still remains unresolved because of some persistent issues that prevent the long-term survival of artificial tissues after implantation. A key factor that is being addressed to improve the in vivo viability of synthetic tissues is the need to promote efficient transport of nutrients and gases to cells within the tissue constructs (Loffredo & Lee, 2008).

The principal transport modality for small molecules within synthetic tissues is passive diffusion of metabolic factors, such as oxygen where the driving force is the concentration gradient across the interstitial matrices. Sufficient oxygen supply is critical for the survival of resident cells. But the oxygen diffusion limit is less than 200 μm. This transport restriction severely constrains approaches to generate viable synthetic tissues of thicknesses greater than 1 cm (Griffith et al., 2005; Loffredo & Lee, 2008; Lovett, Lee, Edwards, & Kaplan, 2009).

In the body, the microcirculation (composed of the microvasculature and lymphatics) plays an important role in delivering gases and nutrients to, as well as removing wastes from, tissues (Loffredo & Lee, 2008; Shiu et al., 2005). In vivo, cells can survive no more than 100 - 200 μm from the nearest capillary (Griffith et al., 2005; Loffredo & Lee, 2008; Lovett et al., 2009). This suggests that, for tissue engineering, generating adequate microcirculation in tissues would be a promising strategy for increasing the success of thick synthetic constructs. Specifically, it would be ideal if tissue engineering strategies could be developed to promote the formation of microvessel networks within artificial tissues that can mimic the in vivo convective transport scheme. One of the biggest obstacles to the success of tissue engineering is to construct sufficient microvascular network in peripheral tissues (Loffredo & Lee, 2008).

In recent years, there have been substantial efforts to design strategies to promote vascularization in artificial tissues. For example, tissue engineered scaffolds embedded with molecules that stimulate formation of blood vessels (angiogenesis) have been developed to encourage in growth of microvasculature from surrounding host tissues after implantation (Lovett et al., 2009; Shiu et al., 2005). In some cases, the cells responsible for angiogenesis have been incorporated into the 3-D matrices of different scaffolds to further aid in microvessel network in growth. Other strategies have been studied to develop microvessels in tissue constructs before their implantation. Such approaches use bioreactors that mainly facilitate nutrients and oxygen delivery to the cells in the tissues to ensure construct viability, but also may be used to prevascularize constructs by aiding transport of angiogenic factors. Finally, there are microelectromechanical systems-related approaches that rely on generating

microvasculature using advanced 3-D microfabrication techniques such as microfluidics (Lovett et al., 2009).

There are also a lot of efforts in developing scaffolds and bioreactors to deliver tubulogenic molecules and apply mechanical forces to resident cells to stimulate microvascular network formation (Freed et al., 2006). For example, it has been reported that low level interstitial flow in 3-D gels could act as a biomechanical factor that enhances and directs cell migration and cell-cell communication (Helm, Zisch, & Swartz, 2007). It has also been found that one can increase formation of neovessel and vessel branching by culturing rat microvessel fragments in collagen gel in the presence of static stretch compared with control group (no stretching) (Krishnan et al., 2008). However, well-controlled distributions of mechanical forces, such as shear stress and tensile stress, are hard to apply to cells embedded in 3-D porous matrices due to the thickness of constructs and complexity of the scaffold/tissue microstructure. On the contrary, pressure seems to be a more readily applicable control parameter to stimulate cells in hydrated porous matrices in order to modulate microvessel formation. Recently, it has been reported that pressure influences endothelial tubulogenic activities, such as cell elongation, proliferation, and fibroblast growth factor-2 (FGF-2) release (Acevedo, Bowser, Gerritsen, & Bizios, 1993). Moreover, a previous study showed that endothelial sprouting is pressure magnitude dependent (Shin, Underwood, & Fannon, 2012). The present study continues to explore the utility of pressure as a tubulogenic stimulus. The following sections will provide the background and rationale of the present study.

1.1 Blood vessels and lymphatic vessels

Blood vessels carry blood throughout the body to deliver nutrients, gases, and biochemical mediators to downstream tissues. The lymphatic vessels are a second transport system that is responsible for the absorption of interstitial fluid from the surrounding tissues to aid in the removal of metabolic waste and to filter pathogens out of the tissue matrices (Rovenska & Rovensky, 2011; Santambrogio & Santambrogio, 2013).

The blood circulation consists of arteries, arterioles, capillaries, venules, and veins. Arteries carry the blood to downstream tissues. These vessels continuously branch into smaller vessels forming networks of arterioles that further branch into capillaries. Capillaries are the smallest diameter and thinnest walled vessels of the vasculature that allow diffusive transport of nutrients, gases, and biological mediators from the blood to enter tissues. They extend into tissues in complex branched networks. Eventually, the capillaries turn into venules that coalesce into veins responsible for bringing back the blood to heart.

Structurally, all blood vessels except capillaries have three layers: tunica intima, tunica media, and tunica adventitia (Shiu et al., 2005). The tunica intima is the inner layer which separates the blood from the wall, and it consists of a layer of endothelial cells, which line the inner surface of the vascular wall in contact with the blood (Shiu et al., 2005). The middle layer is called tunica media and is made of alternating layers of elastin fibers and smooth muscle cells. And the outermost layer is tunica adventitia, which is entirely made of connective tissue (Shiu et al., 2005).

In contrast to the structure of arteries and veins, capillaries have a more simplistic structure, which provides minimal barriers to blood-to-tissue transport. These smallest of vessels only consist of a layer of endothelial cells on a basal lamina (Carmeliet, 2003).

Abnormal capillary growth leads to pathological conditions. The excessive formation of blood vessels supports the growth of diseased tissues and the newly formed vessels are usually leaky. The excessive blood vessel growth causes disorders such as during the pathobiology of cancer, obesity (Rupnick et al., 2002), atherosclerosis, and diabetic nephropathy (Carmeliet, 2003). On the other hand, insufficient vessel growth leads to tissue ischemia and organ failure (Carmeliet, 2003). Adequate capillary network generation is essential for healthy tissue growth in terms of supplying nutrients to tissues.

The major functions of lymphatic network are maintaining blood and tissue volume, absorbing and delivering dietary lipids from the intestine to the liver, and trafficking immune cells (Santambrogio & Santambrogio, 2013). Moreover, the lymphatics are responsible for draining excess interstitial fluid and preventing the accumulation of metabolic wastes (Rovenska & Rovensky, 2011).

There are two segments of lymphatic network: initial lymphatics and collecting lymphatics (or contractile lymphatics). The initial lymphatics are located inside the tissue parenchyma and consist of a layer of endothelial cells without smooth muscle media, while the collecting lymphatics have smooth muscle media (Mendoza & Schmid-Schonbein, 2003; Trzewik, Mallipattu, Artmann, Delano, & Schmid-Schonbein, 2001). Initial lymphatics, typically referred to as lymphatic capillaries, are responsible for collecting and transporting interstitial fluid, proteins, colloids, and cells (Schmid-

Schonbein, 2003). Several initial lymphatics connect together forming a contractile lymphatic vessel. Contractile lymphatics undergo peristaltic contractions (Schmid-Schonbein, 2003).

Insufficient lymphatics cause lymphedema due to the interstitial accumulation of fluid. Besides promoting tissue swelling due to the accumulation of interstitial fluid, which contains high amount of protein, insufficient lymphatics also cause inflammatory reaction and abnormal adipose tissue deposition (Chakraborty, Gurusamy, Zawieja, & Muthuchamy, 2013). On the other hand, advanced tumors have been reported to be associated with upregulation of lymphatics formation which is thought to facilitate the entry of metastatic cancer cells into the blood stream and where they may spread to other tissues and organs (Cao, 2008).

1.2 Endothelial cells

All the blood vessels have at least one layer of endothelial cells. Endothelial cells play many important roles in synthetic and metabolic processes including blood pressure regulation, blood coagulation and fibrinolysis, vascular tone, adhesion and transmigration of inflammatory cells, and tubulogenesis (Bouis, Hospers, Meijer, Molema, & Mulder, 2001; Michiels, 2003; Sumpio, Riley, & Dardik, 2002). In these processes, endothelial cells respond to the changes in local physiological conditions (Shiu et al., 2005).

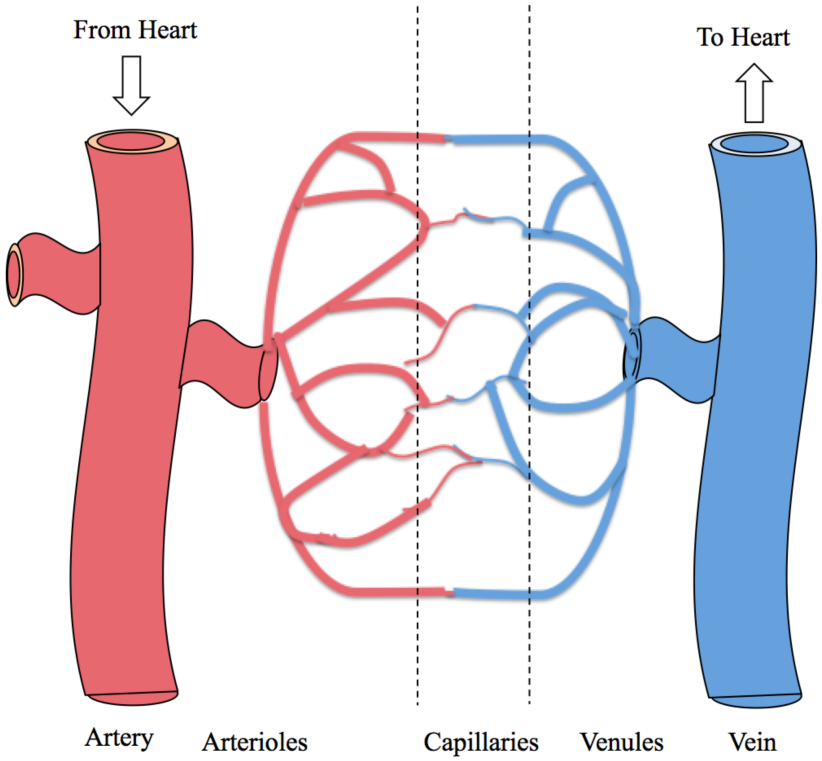

Figure 1.1. Diagram of blood vessel network. Figure adapted from 2006 Encyclopedia Britannica, Inc..

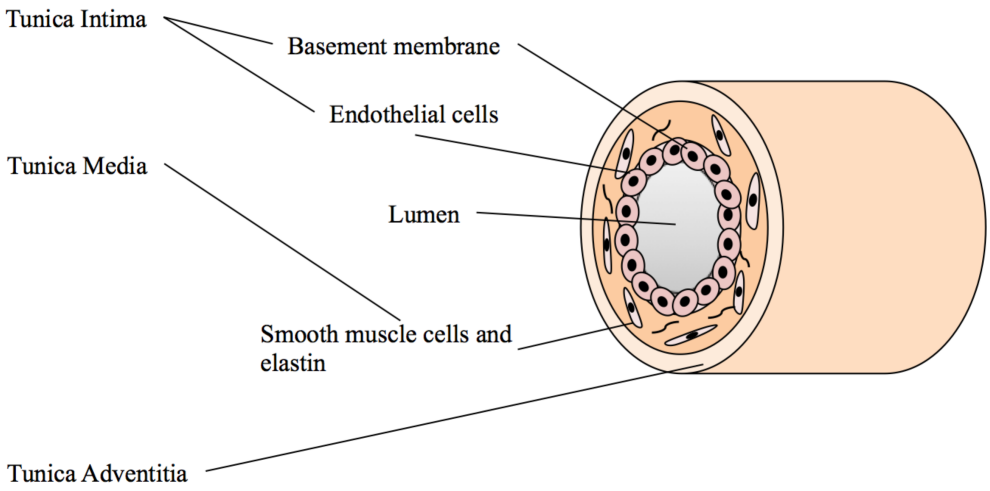

Figure 1.2. Diagram of artery three-layer structure.

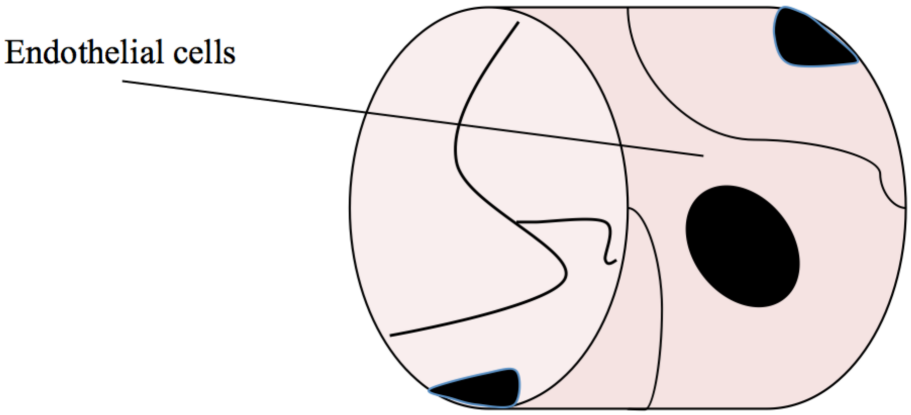

Figure 1.3. Diagram of capillary structure.

Endothelial cells derive from endothelial progenitor cells. They differentiate into different endothelial phenotypes (e.g., large vessel vs. microvascular; arterial vs. venous; capillary vs. arteriolar vs. venular; vascular vs. lymphatic, etc.) due to anatomical location and their surrounding environmental conditions (Carmeliet, 2003). The various types of endothelial cells reside in areas that differ in local blood pressures and vascular wall compositions, which may contribute to the site-specificity of their phenotype. The heterogeneity of endothelial cells is demonstrated by their differential expression and responsiveness to angiogenic factors, such as VEGF or Ang-1, in different tissues. For example, Ang-1 stimulates angiogenesis in skin but has inhibitory effect in the heart (Carmeliet, 2003; Visconti, Richardson, & Sato, 2002).

Endothelial cells are the inner, blood-contacting surface of the blood vessels. Because of the physiological environment, the endothelial cells experience three external mechanical forces: hydrodynamic pressure and shear stress caused by the blood flow, and also the tensile stress which is muscle contraction or vessel compliance (Shiu et al., 2005). Mechanical stimulation alters endothelial cell activity including intracellular signaling, gene expression, and protein expression (Chien, 2007; Shiu et al., 2005). Endothelial cells may also invade tissues during tubulogenic processes where they may experience similar types of mechanical stimuli, but of different magnitudes and modalities.

The processes of blood vessel formation, particularly capillaries are mostly initiated and mediated by endothelial cells. These processes involve proliferation and migration of endothelial cells that are regulated by a complex array of biochemical factors such as vascular endothelial growth factor-A (VEGF-A) (Hicklin & Ellis, 2005),

vascular endothelial growth factor-C (VEGF-C) (Hoeben et al., 2004), and fibroblast growth factor-2 (FGF-2) (Burgess & Maciag, 1989), as well as mechanical factors including shear stress (Goldman et al., 2007), solid matrix tension (Davis & Camarillo, 1995), and pressure (Acevedo et al., 1993).

1.3 Tubulogenesis

Tubulogenesis refers to the general process of new vessel formation from pre-existing vessels. There are three types of tubulogenesis carried out by endothelial cells: vasculogenesis, angiogenesis, and lymphangiogenesis.

Vasculogenesis describes the de novo formation of primitive vasculature from endothelial cell precursors, particularly during embryogenesis (Shiu et al., 2005). Angiogenesis refers to the formation of blood vascular capillaries (Shiu et al., 2005), and lymphangiogenesis is the formation of lymphatic capillaries. Angiogenesis and lymphangiogenesis occur during post-developmental processes (e.g., wound healing, tissue remodeling, etc.). Both of these can be described as following a similar progression of endothelial cell mediated processes. Whether a particular tubulogenic activity is angiogenic or lymphangiogenic depends on the phenotype of the endothelial cells that mediate it. Microvascular endothelial cells generate the blood capillaries, while the lymphatic endothelial cells form lymphatic capillaries.

There are two types of angiogenesis: intussusceptive angiogenesis and sprouting angiogenesis (Adair & Montani, 2010; Shiu et al., 2005). Intussusceptive angiogenesis is also called “splitting angiogenesis”. It is a process of one single vessel splitting into two

daughters and ultimately forming a branch (Adair & Montani, 2010; Shiu et al., 2005). Intussusceptive angiogenesis is primarily responsible in expanding the size of capillary networks and increasing their complexity (Burri, Hlushchuk, & Djonov, 2004). This process does not require intense proliferation of endothelial cells, but more focuses on the rearrangement of endothelial cells (Kilarski & Gerwins, 2009). The intussusceptive angiogenesis process is usually seen during wounding healing, tumor vascularization, and growth of endometrium during the female menstruation cycle (Kilarski & Gerwins, 2009). But it is not known how split vessels move into avascular areas during healing of wounds (Kilarski & Gerwins, 2009).

Sprouting angiogenesis depends on endothelial invasion of tissues involving matrix degradation, proliferation and migration (Adair & Montani, 2010). During

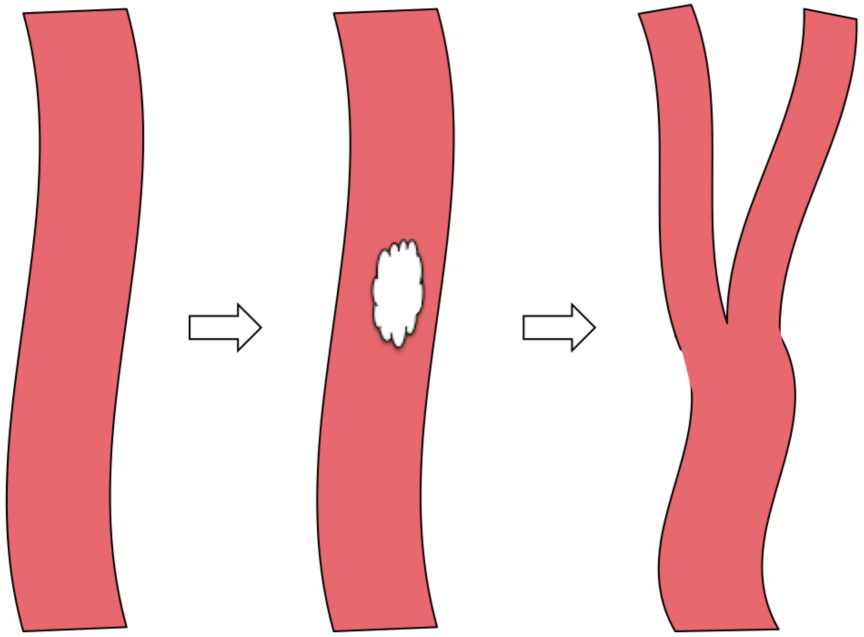

Figure 1.4. Diagram of intussusceptive angiogenesis.

sprouting angiogenesis, the endothelial cells are recruited and activated by angiogenic growth factors, such as fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs), or angiopoietins (Angs) (Ucuzian & Greisler, 2007). They start to proliferate and migrate, and they secrete proteolytic enzymes to degrade and remodel local extracellular matrix. Finally, the endothelial cells differentiate and sprout into linear cord-like structures. These cord-like structures elongate due to proliferation and lastly form lumens (Shiu et al., 2005). It is also believed that other cells such as pericytes, fibroblasts, and smooth muscle cells are recruited to support the later stages of sprouting angiogenesis by forming the basement membrane around the new vessel (Shiu et al., 2005; Ucuzian & Greisler, 2007).

Lymphangiogenesis is a process that describes the formation of new lymphatic capillaries from pre-existing lymphatics. The process of lymphangiogenesis is thought to be similar to angiogenesis. And it involves the activation, migration, sprouting, and proliferation of lymphatic endothelial cells (Ji, 2009; Stacker et al., 2014). The proliferation and migration of lymphatic endothelial cells depend on vascular endothelial growth factor receptors (VEGFR); specifically VEGFR-2 and VEGFR-3 (Ji, 2009; Stacker et al., 2014). They bind to vascular endothelial growth factor C (VEGF-C) or VEGF-D (Ji, 2009; Stacker et al., 2014). Fibroblast growth factor 2 (FGF-2), which has been shown to promote angiogenesis, has also been reported to have the ability to synergize with VEGF-C to stimulate lymphangiogenesis by endothelial cells (Stacker et al., 2014).

1.4 Tubulogenic Growth factors

The formation of a microvascular network requires delicate coordination of not only different cells such as fibroblasts, smooth muscle cells, and endothelial cells, but also the expression of tubulogenic molecules, such as FGFs, VEGFs, ephrinB2, and Ang-1 (Bouis et al., 2001; Michiels, 2003; Shiu et al., 2005; Stacker et al., 2014; Ucuzian & Greisler, 2007). Among these, the influences of the FGFs and VEGFs have received widespread attention. The involvements of tubulogenic molecules that fall within these two families of growth factors were a focus of the present study.

Fibroblast Growth Factor (FGF) family

FGF was identified as a polypeptide mitogen from bovine pituitary and later from brain (Slavin, 1995). To date, there are at least 20 FGFs and 4 fibroblast growth factor receptors (FGFRs) recognized (Cross & Claesson-Welsh, 2001; Sumpio et al., 2002). FGFs not only act on endothelial cells, but also some other cells such as fibroblasts and pericytes (Shiu et al., 2005). They are involved in tissue homeostasis, cell proliferation, migration, and differentiation (Boilly, Vercoutter-Edouart, Hondermarck, Nurcombe, & Le Bourhis, 2000; Cross & Claesson-Welsh, 2001).

FGF-1, or acidic FGF (aFGF), is an anionic mitogen and mostly found in neural tissue. In comparison, FGF-2 is more widely present in many tissues such as brain, kidney, adrenal, and corpus luteum (Slavin, 1995). Importantly, FGF-2 is found in basement membrane of every blood vessel from all the organs (Slavin, 1995). FGF-2, also known as basic FGF (bFGF), is capable of promoting angiogenesis (Cross & Claesson-Welsh, 2001; Shiu et al., 2005; Thisse & Thisse, 2005), and lymphangiogenesis

(Cao et al., 2012).

The four FGFRs are FGFR-1, FGFR-2, FGFR-3, and FGFR-4 (Boilly et al., 2000; R. Cao et al., 2012; Cross & Claesson-Welsh, 2001; Shiu et al., 2005). These FGFRs mediate the biological effects of FGFs (Boilly et al., 2000; Cross & Claesson-Welsh, 2001). FGF-2 binds to FGFR-1 (Boilly et al., 2000; R. Cao et al., 2012; Cross & Claesson-Welsh, 2001), and studies have showed that blockade of FGFR-1inhibits FGF-2 induced tubulogenesis (Boilly et al., 2000; R. Cao et al., 2012). It has been reported that FGFR-1 is involved in vessel formation and maintenance in the embryo (Lee, Schloss, & Swain, 2000). FGF-2 also binds to FGFR-2, which promotes some endothelial tubulogenic activities, such as cell migration, proliferation, and matrix protease production (Slavin, 1995).

FGF-2 promotes many tubulogenesis processes involving endothelial proliferation, basement membrane degradation, and migration. FGF-2 promotes DNA synthesis and cellular division in many cell types, including endothelial cells, fibroblasts, and smooth muscle cells (Burgess & Maciag, 1989). Moreover, it has been reported that interstitial collagenases produced by endothelial cells may be induced by FGF-like molecules, and these collagenases play a role in the invasive process during angiogenesis (Mignatti, Tsuboi, Robbins, & Rifkin, 1989; Moscatelli, Presta, Joseph-Silverstein, & Rifkin, 1986). Finally, FGF-2 induces bovine capillary endothelial cell invasion into basement membrane (Mignatti et al., 1989). Endothelial migration depends expression of integrins, such as $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_v\beta_5$ integrins, which are upregulated by FGF-2 stimulation. Moreover, FGF-2 treated endothelial cells exhibit high affinity for fibronectin, laminin, vitronectin, and type I collagen, proteins that make up the

extracellular matrices on which these cells migrate (Klein et al., 1993). This suggests that FGF-2 is sequestered in the extracellular matrices where they may be released due to tissue damage or remodeling.

Interestingly, FGF-2 is also involved in lymphatic endothelial cell proliferation and migration. It has been reported that FGF-2 is involved in the expression of lymphangiogenic growth factor VEGF-C by cultured endothelial cells (Skobe & Detmar, 2000). Furthermore, VEGF-C/VEGFR-3 signaling is essential for FGF-2 stimulated lymphangiogenic activities (R. Cao et al., 2012). In fact, FGF-2 induced lymphangiogenesis is dose-dependent. Specifically, FGF-2 stimulates lymphangiogenesis at low concentrations, but it promotes angiogenesis at high concentrations (Chang et al., 2004).

Vascular Endothelial Growth Factor (VEGF) family

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999; Ucuzian & Greisler, 2007). There are six members in the VEGF family of growth factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PIGF), and the orf parapoxvirus VEGF (VEGF-E) (Cross & Claesson-Welsh, 2001). The biological effects of VEGFs are mediated by three tyrosine-kinase receptors: VEGFR-1, VEGFR-2, and VEGFR-3 (Cross & Claesson-Welsh, 2001; Neufeld et al., 1999). The binding relationships between the VEGFs and VEGFRs are shown in table 1.1. The expression of VEGFR-1 and VEGFR-2 mostly

occurs in endothelial cells, while VEGFR-3 expression is restricted, for the most part, to lymphatic endothelial cells (Cross & Claesson-Welsh, 2001; Hoeben et al., 2004).

VEGF-A plays a role in both physiological and pathological angiogenesis including angiogenesis in tumor and intraocular syndromes (Ferrara, 2001). It has been reported that VEGF-A is required for endothelial cell survival (Ferrara, 2001). VEGF-A also plays a role in regulation of microvascular permeability (Ferrara, 2001). Permeability has been proposed to be important in angiogenesis, because the leaked plasma proteins and the formation of a fibrin gel promotes the proliferation of endothelial cells (Ferrara, 2001). VEGF-A binds to VEGFR-1, also known as fms-like tyrosine kinase 1 (Flt-1), and VEGFR-2, also known as KDR, and the murine homologue (Flk-1) (Hicklin & Ellis, 2005). VEGFR-1 is critical in angiogenesis, but its impact on endothelial cells, upon ligand binding, is weak in comparison with that of VEGFR-2. VEGF-A/VEGFR-2 stimulates angiogenic processes including microvascular permeability, cell proliferation, migration, and invasion (Hicklin & Ellis, 2005).

Two key molecules of interest to the present study are VEGFR-3, also known as fms-like tyrosine kinase 4 (Flt-4), and its ligand VEGF-C. VEGF-C is found mostly in heart, placenta, ovary, small intestine, and the thyroid gland (Hoeben et al., 2004) and is expressed by endothelial cells including bovine aortic endothelial cells (BAECs) (Shin et al., 2012). VEGF-C is mainly linked to lymphangiogenesis (Hoeben et al., 2004) although it is reported to have angiogenic effects. For example, VEGF-C stimulates endothelial angiogenic activities even after blocking VEGFR-2 signaling pathways (Persaud et al., 2004). By excluding the possibility of VEGF-A/VEGFR-2 and VEGF-C/VEGFR-2 signaling, Persaud et al. still showed VEGF-C may enhance angiogenic

activity of endothelial cells which indicated a role for VEGF-C in angiogenesis (Persaud et al., 2004). VEGFR-3 is the primary receptor of VEGF-C (Tammela et al., 2008) although it also has affinity for VEGF-D.

Table 1.1. VEGF family and their receptors. Summary of binding of VEGF family members to VEGFRs.

	VEGFR-1	VEGFR-2	VEGFR-3
VEGF-A	✔	✔	✗
VEGF-B	✔	✗	✗
VEGF-C	✗	✔	✔
VEGF-D	✗	✔	✔
VEGF-E	✗	✔	✗
PIGF	✔	✗	✗

For the most part, VEGFR-3 is expressed mainly in lymphatic endothelial cells with the ability to stimulate cell proliferation, cell migration, and apoptosis (Hoeben et al., 2004). But VEGFR-3 is also expressed by vascular endothelium such as BAECs, human umbilical vein endothelial cells (HUVECs), and human microvascular endothelial cells (HMVECs) (Kashima et al., 2012; Pepper, Mandriota, Jeltsch, Kumar, & Alitalo, 1998; Persaud et al., 2004). In this study, we aimed to investigate the involvement of

VEGFR-3 in pressure-induced endothelial tubulogenesis, so we used an indolinone called MAZ51 to inhibit activity of VEGFR-3. It has been reported that MAZ51 has the ability to block the activity of VEGFR-3 at low concentrations (< 20 μM), and inhibits VEGFR-2 at concentrations higher than 20 μM (Kirkin et al., 2004; Lin et al., 2008).

1.5 Mechanotransduction

Because endothelial cells in capillaries are adjacent to the blood flow, they are subjected to fluid shear stresses, tensile stresses due to pulsatile matrix deformation, and hydrodynamic pressures (Chien, 2007; Shin et al., 2002; Shiu et al., 2005). In interstitial tissues where sprouting angiogenesis occurs, the endothelial cells may also be exposed to the interstitial pressures and flows. It has been found that the tubulogenic activities of endothelial cells are subjected to regulation by the mechanical stimuli (Shiu et al., 2005). Fluid shear stress has been reported to be involved in angiogenic processes by regulating VEGF-A expression (Gan, Miocic, Doroudi, Selin-Sjogren, & Jern, 2000; Zheng, Seftor, Meininger, Hendrix, & Tomanek, 2001). Endothelial cells respond to shear stress through intracellular signaling, gene expression, and protein expressions (Chien, 2007). It has been reported that low shear stress caused by interstitial flow regulates endothelial morphology changes; for example, lymphatic endothelial cells were elongated, while the blood endothelial cells were branched and formed lumens (Ng, Helm, & Swartz, 2004). On the other hand, high shear stress leads to endothelial cell remodeling (Ng et al., 2004). Tensile stress caused by stretching regulates neovascular sprouting and elongation (Krishnan et al., 2008). It has also been reported that solid matrix tension plays a role in

endothelial cell migration (Davis & Camarillo, 1995).

Compared to shear stress and stretch, pressure has only received limited attention in terms of its effects on endothelial tubulogenic activities such as elongated endothelial morphological changes (Acevedo et al., 1993; Schwartz, Bizios, Medow, & Gerritsen, 1999; Sumpio, Widmann, Ricotta, Awolesi, & Watase, 1994), increased endothelial proliferation (Acevedo et al., 1993; Schwartz et al., 1999; Sumpio et al., 1994), and enhanced endothelial sprout formation (Shin et al., 2012). There is also evidence that pressure-induced endothelial cell activities involve increased release of cytoplasmic FGF-2 (Acevedo et al., 1993) and expression of VEGF-C (Shin et al., 2002). This data implicates pressure is involvement in both angiogenesis and/or lymphangiogenesis processes.

Notably, there are pathological conditions involving dysregulated tubulogenic activities that are also associated with high pressure. Severe pulmonary hypertension (>25 mmHg) is often associated with pulmonary arterial remodeling, where the intimal endothelial cells adopt a precapillary-like phenotype (Tuder & Voelkel, 2002; Yeager, Halley, Golpon, Voelkel, & Tuder, 2001). Patients who have cigarette smoking-induced emphysema and have a high pulmonary artery pressure (35 – 40 mmHg) are also found to have reduced number of peripheral lung blood vessels (Tuder & Voelkel, 2002). Furthermore, it has been found that the interstitial fluid pressure is over 50 mmHg in tumors, which is associated with tumor metastasis (Boucher & Jain, 1992). Notably, there are reports that tumors with interstitial hypertension (up to 50 mmHg) are associated with upregulated VEGF-C expression and are at more risk for metastasis (Y. Cao, 2008; Nathan et al., 2008). Presumably, the enhanced VEGF-C expression promotes lymphatic

formation in tumors, which facilitates the ability of metastatic cancer cells to get into the blood stream and spread to other organs with deadly consequences (R. Cao et al., 2012; Nathan et al., 2008). These in vivo data suggest a link between interstitial pressure levels and the state of endothelial tubulogenic activity.

Recently, it was reported that pressure is, in fact, a magnitude-dependent stimulus for 1) cell proliferation that depends on VEGFR-3 and 2) sprout formation by endothelial cells grown on Cytodex microcarrier beads in three-dimensional collagen gels (Shin et al., 2012). The present study was carried out to further examine the potential influence of pressure on endothelial sprouting.

1.6 Rationale

There is a growing interest to investigate the effects of mechanical forces such as shear stress and tensile stress on endothelial tubulogenesis, but the effects of hydrostatic pressure are mostly neglected even though endothelial cells are exposed to different pressure levels during microvascular network remodeling under physiological and pathological conditions. The present study investigates a potential connection between interstitial pressure fluctuations and tubulogenic activities of endothelial cells. Along this line, the present study addresses the hypothesis that endothelial tubulogenic activities are pressure magnitude and exposure time dependent involving VEGFR-3 activity.

Previously, our lab showed that endothelial tubulogenesis is pressure-magnitude dependent (Shin et al., 2012). This study was based on a microcarrier bead model, and studied endothelial tubulogenesis when exposed to 0, 20, or 40 mmHg. But it only

examined sprout formation after three days of exposure to only two pressure levels, which is insufficient to assess how pressure-sensitive endothelial sprouting is. The minimum exposure times and magnitudes required to have an effect on endothelial tubulogenic activity are unknown. Moreover, the study in question had used a three-dimensional microbead model that may be a challenge to translate to tissue engineering applications because the beads are made of non-biodegradable material.

The present investigation sought to adapt the use of endothelial spheroid cultures into our study. Endothelial spheroids are cellular aggregates without the need for a non-physiologic substrate for supporting endothelial cell growth and viability. Simultaneously, we aimed to identify the operating pressures (0 – 40 mmHg) and exposure times for controlling endothelial sprouting rates, and also the contribution of VEGF-C/VEGFR-3 signaling pathway in the presence of pressure. For this purpose, we first exposed BAEC spheroids to 20 mmHg for 3 days and compared with their responses to those of endothelial spheroids cultured under atmospheric conditions. The goal of this first set of experiments was to test if endothelial cells in spheroid cultures exhibited similar sprout activity to cells in beads.

We also aimed to identify the minimum exposure time required for endothelial spheroid cultures to display a change in their sprouting activity under 20 mmHg. Then we studied the sprouting responses of endothelial spheroids exposed the spheroids to 5 mmHg and 40 mmHg for 2 and 3 days. For these experiments, the goal was to examine the effects of different pressure levels and exposure times on endothelial sprouting (i.e., pressure level dependence and time dependence).

Finally, we aimed to study the dependence of pressure-induced endothelial sprouting on VEGFR-3 signaling. We chose to conduct these studies with spheroids exposed to 20 mmHg and in the presence of VEGFR-3 blocker, MAZ51.The goal was to determine whether VEGFR-3 signaling plays a role in pressure-sensitive sprout formation in support of prior studies reporting evidence that exposure to 20 mmHg for 3 days stimulates endothelial proliferation involving autocrine VEGF-C/VEGFR-3 signaling. This has implications in determining if pressure may be used as a way to control the endothelial tubulogenic phenotype (angiogenesis vs. lymphangiogenesis).

It is anticipated that the results from this study would provide insight into the physiological regulation of endothelial capillary formation during health and disease as well as the potential use of hydrostatic pressure as a tissue engineering control parameter for microvascularizing synthetic tissues.

2. Materials and Methods

2.1 Cell culture

Bovine aortic endothelial cells (BAECs; Invitrogen) were cultured in 25-cm^2 cell culture flasks (T-25; BD Falcon) in basal Dulbecco's Modified Eagle Medium (DMEM; Hyclone) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone), 1% penicillin/streptomycin/L-glutamine solution (P/S/G; Hyclone). This DMEM formulation will heretofore be referred to as complete medium. For routine culture, the cells were maintained at 37 °C in the 5% carbon dioxide/95% air environment of a standard tissue culture incubator, and the complete medium was exchanged every 2 to 3 days.

Upon reaching greater than 90% confluence, cell populations were split at ratios of either 1:2 or 1:3 v/v. Specifically, BAECs were rinsed with 5 mL of phosphate buffered saline (PBS; MP Biomedicals) for 1 or 2 minutes and then the PBS was aspirated. Cells were dissociated from substrates by incubation in 0.05% trypsin (Fisher Scientific) with periodic gentle agitation for 1 to 2 minutes. When all the cells were detached from the substrates, 3 mL of complete medium was added to the flask to deactivate the trypsin. The cell suspensions were then subcultured into 2 (1:2 split) or 3 (1:3 split) fresh tissue culture flasks. In the present study, cells of up to 15 passages were used for experiments.

2.2 Cell Storage

For long-term storage, cell populations that were greater than 90% confluent in T-25 flasks were rinsed with 5 mL of PBS for 1 to 2 minutes and then lifted by incubation in 1 mL of 0.05% trypsin for 1 to 2 minutes in the incubator. After complete cell detachment from the flask surfaces, 3 mL of complete medium was added into the flask to deactivate the trypsin. The cell solution was transferred to a 15-mL centrifuge tube and pelleted at 200xg centrifugation for 5 minutes at 4 °C. The cells were resuspended in 1.5-mL of sterile-filtered 10% dimethyl sulfoxide (DMSO; Sigma) in FBS. The cells/DMSO/FBS solution was transferred to 2-mL cryogenic vials (BD Falcon), placed at -80 °C for 1 day, and then transferred to liquid nitrogen for cryogenic storage.

2.3 Thaw cells

Frozen cell cultures were thawed quickly by partially submerging the 2-mL cryogenic vial in a 37 °C water bath with gentle agitation. When the cell solution was thawed (within 2 to 3 minutes), it was transferred to a new T-25 flask containing 3.5 mL of warm complete media and allowed to culture in a standard incubator for 1 day. After this initial day of culture, the supernatant containing non-adherent (non-viable) cells was removed and replaced with fresh complete media.

2.4 Methocel preparation

Complete media containing high viscosity (4000 centipoise) methyl cellulose (ACROS Organics) was used for endothelial spheroid generation (see section 2.5). To prepare this methocel solution, 0.48 g methyl cellulose powder was added to a 100-mL glass bottle containing a stir bar and subsequently autoclaved. A 20-mL aliquot of basal DMEM preheated to 60 °C was added to the autoclaved methyl cellulose and subjected to agitation on a magnetic stir plate for 20 minutes at room temperature. At this time, an additional 20 mL of basal DMEM was added to the bottle and mixed at 4 °C for 1 to 2 hours. The final solution containing 12 mg/mL methocel was then cleared by centrifugation (2500xg, 2 h, at room temperature), and the clear supernatant, representing the stock methocel solution to be used for spheroid generation, was stored at 4 °C.

2.5 Spheroid generation

To generate spheroids of defined sizes, i.e., cell numbers, we used a hanging droplet method (Figure 2.1) that was adapted from the literature (Korff & Augustin, 1998). Confluent monolayers of BAECs were trypsinized/detached from T-25 flask surfaces and resuspended in complete DMEM. The densities of the cell suspensions were determined using a hemocytometer. BAECs at a density of 45 cells/µL were homogenously suspended in complete media containing 2.4 mg/mL methocel stock solution (see section 2.4). To make spheroids, a micropipette was used to deposit 15-µL

droplets of this cell mixture in a sterile petri dish. Each petri dish held up to 90 droplets. The petri dishes with the droplets were then flipped over 180^{o} onto their sterile lids so that droplets were hanging upside down (Figure 2.1). The droplets were cultured in a standard cell culture incubator environment for 24 hours. Each droplet culture formed one spheroid.

2.6 Three-dimensional spheroid sprouting assay

2.6.1 Collagen solution formulation

A gel solution containing 2.25 mg/mL Collagen type I was prepared according to the following formula: 80 parts 10X DMEM (Sigma), 10 parts 200 mM L-glutamine (Invitrogen), 549 parts Collagen type I (4.1 mg/mL; Corning), 13 parts 1 N sodium hydroxide solution (NaOH), 50 parts 0.53 N sodium bicarbonate solution ($NaHCO_3$), 238 parts methocel stock solution, and 60 parts DMEM. All these reagents were added in the order described and involved thorough mixing. Moreover, the steps carried out to make the gel solution were conducted on ice (i.e., at 4^{o}C) using pre-cooled reagents and tubes.

2.6.2 Harvest spheroids

Endothelial spheroids were harvested from petri dishes and suspended at a density of 18 spheroids/mL of PBS. These spheroid suspensions were then transferred to 15-mL centrifuge tubes and pelleted at 200xg for 3 minutes. The supernatants were aspirated, and the spheroids were then resuspended in the gel solution.

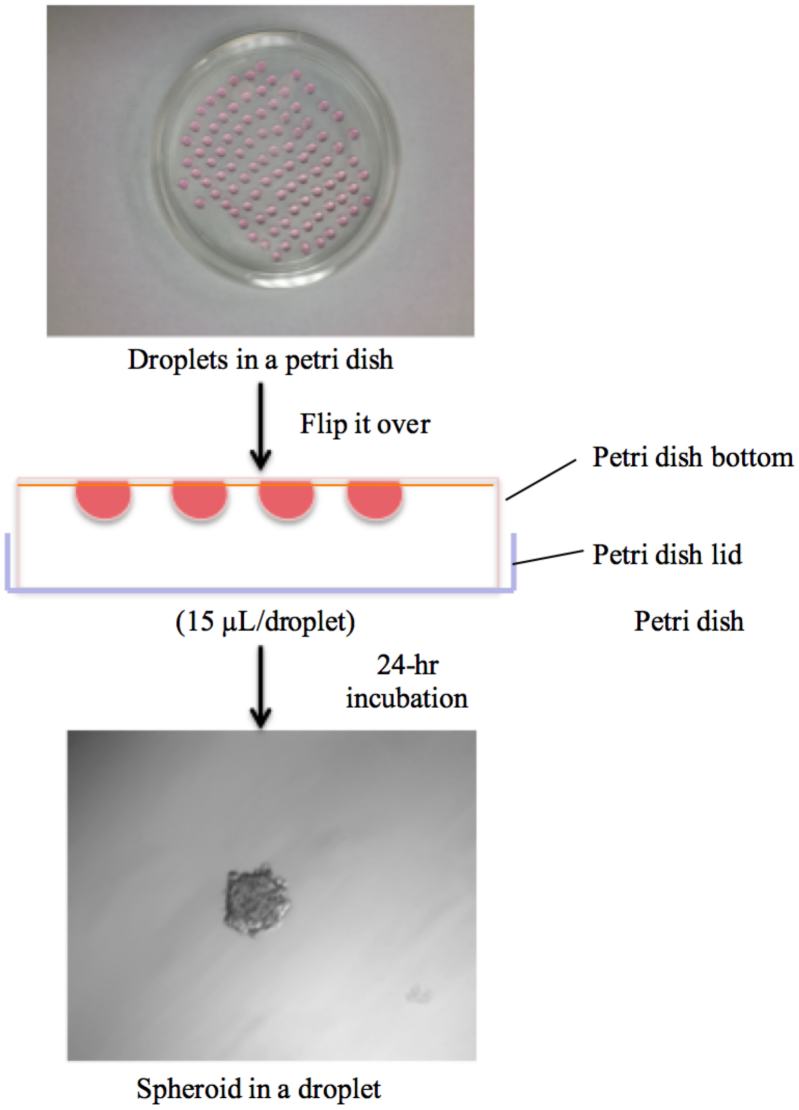

Figure 2.1. Schematic of the process used to generate endothelial spheroids. Endothelial spheroids were prepared using BAECs suspended in 15-µL droplets containing 20% methocel stock solution at a cell density of 45 cells/µL. Droplets were plated in a petri dish and cultured upside down in the incubator for 24 hours.

2.6.3 Gel polymerization

Three-dimensional (3-D) collagen gels with spheroids were generated following procedures adapted from a previous study (Shin et al., 2012). Briefly, aliquots (50 μL) of acellular collagen gel solution was deposited in individual wells of a 96-well tissue culture plate and allowed to polymerize for 20 minutes in a standard cell culture incubator. A second aliquot (50 μL) of gel solution, but this time, containing endothelial spheroids (450 spheroids/mL) was then deposited on top of the initial gel layer and allowed to polymerize for another 20 minutes. After polymerization, 50 μL of complete media were added to each well. In some gel cultures, the complete media was supplemented with either 0.5 μM MAZ51 (Millipore; VEGFR3-selective, tyrosine kinase inhibitor) in DMSO or 0.01% DMSO (Sigma; vehicle control).

2.7 Pressure Exposure

A custom pressure system (Figure 2.3) was used to expose endothelial cell preparations to stable hydrostatic pressures in a sealed chamber as previously reported (Shin et al., 2012). The pressure system consists of 8 major components: a compressed gas tank, a water column, a humidifier, a polycarbonate pressure chamber, a temperature-controlled oven, pressure transducer, laptop with LabVIEW, and various interconnecting tubing.

Figure 2.2. Schematic depicting the generation of 3-D collagen gels containing endothelial spheroids. Figure adapted from (Shin et al., 2012). An acellular bottom layer collagen gel solution was allowed to polymerize first in the incubator for 20 minutes. A second top layer containing the spheroids was then deposited on the bottom layer and allowed to polymerize in the incubator for another 20 minutes.

The pressure chamber and the humidifier were maintained at 37 °C in a temperature-controlled oven. The polycarbonate pressure chamber was designed to contain one cell culture plate. There are three ports on the pressure chamber: one connected to the compressed gas through a humidifier, one connected to the pressure transducer and pressure monitored by LabVIEW, and the other one connected to the resistance tubing.

A compressed 5% CO_2/95% air mixture in a gas cylinder was delivered to the pressure chamber to generate the hydrostatic pressure used to stimulate the cells. The flow of the compressed gas from the gas cylinder was split to two gas flows. One of them fed a down tube with its tip submerged down a hydrostatic water column. The other branch fed a humidifying chamber (to hydrate the gas) before injection into the sealed pressure chamber. The gas was allowed to pass through the chamber and exit to the atmosphere through resistance tubing. The resistance tubing was responsible for the development of a pressure in the pressure chamber.

The pressure generated in the pressure chamber was controlled by adjusting the depth of the down-tubing submerged in the water column; i.e., the pressure in the pressure chamber equals to the hydrostatic pressure generated in the water column. A pressure regulator attached to the gas tank controlled the flow rate of the compressed gas that went into the system. In order to save gas, the flow rate was set to a minimum value to ensure the amount of gas entering the system was enough to support the desired pressure levels and cell viability. The resistance tubing was used to adjust the outflow rate. The pressure chamber was connected to the pressure transducer that interfaced to a

multi-channel analog to digital converter. The real-time and continuous pressure level in the chamber was monitored using LabVIEW software on a computer.

For pressure experiments, 96-well tissue culture plates containing gel cultures were placed in the sealed chamber of our custom system, and the endothelial spheroids were exposed to 5, 20, and 40 mmHg sustained hydrostatic pressures for time periods of 2 or 3 days. Controls were parallel spheroid cultures maintained under atmospheric pressure in a standard cell culture incubator.

2.8 Fixation and staining

Stock 8 (w/v)% para-formaldehyde (p-form) solution was prepared by dissolving 0.8 g p-form (Acros Organics) in 10 mL deionized water (diH_2O) with gentle agitation at 85 ^oC until it turned clear. To aid this dissolving process, drops of 1 N NaOH were added intermittently during the stirring process until the p-form stock solution clarified. The stock 8% p-form solution was then filtered through a 0.2 μm filter, placed in tube wrapped in foil (to avoid light exposure), and stored in the refrigerator (4 °C). The fixative reagent used for gel assays was prepared by combining 2.5 mL of the stock 8% p-form solution with 0.625 mL of 8% glutaraldehyde (Electron Microscopy Sciences) and 5 mL of 0.2M phosphate buffer (Sorenson's formulation; pH 7.4), and 1.875 mL of diH_2O.

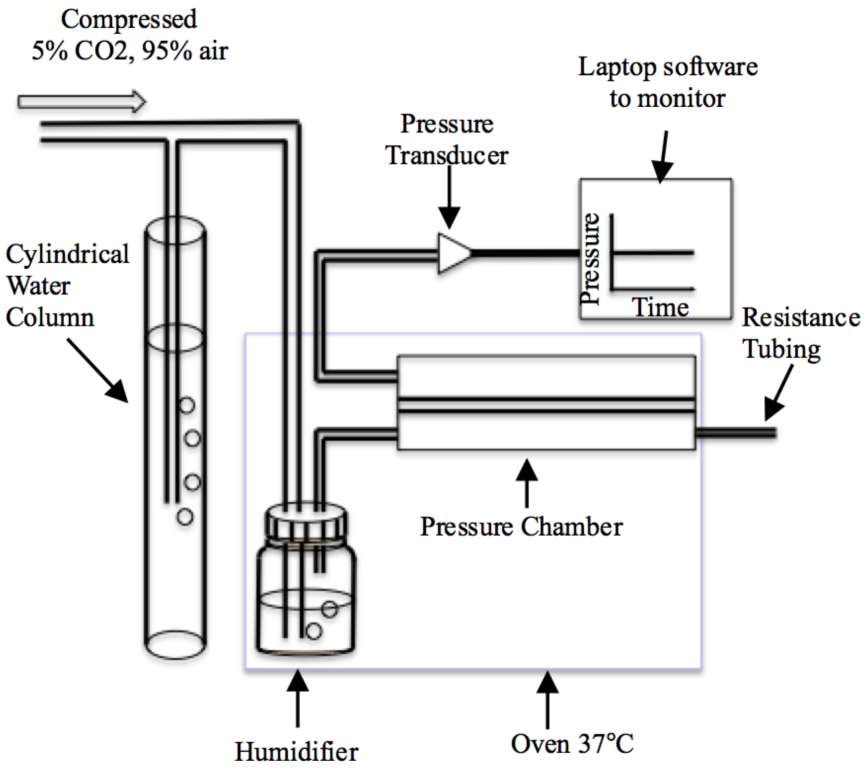

Figure 2.3. Custom hydrostatic pressure system. Figure adapted from (Shin et al., 2012). A compressed gas tank was used to deliver pressurized 5% CO_2 and 95% air environment to a downstream pressure chamber maintained at 37 °C in a temperature-controlled oven. Cells in the pressure chamber were exposed to desired hydrostatic pressure levels using a hydrostatic water column setup connected between the gas tank and the pressure chamber. Controls were cells maintained at atmospheric pressure in a standard incubator.

To fix 3-D spheroid cultures, the media above the gels were gently removed using disposable (plastic) transfer pipettes and replaced with 100 µl of cold fixative reagent. The gels with fixative were then incubated in the dark at 4 °C for 2 hours. The gels were then washed with 1X PBS for 10 minutes at 4 °C and permeablized with 0.1% triton X-100 in PBS for 30 minutes at 4 °C. After this step, the gels were washed with 1% BSA in PBS three times and immediately stained with 2 µg/mL 4′6-diamidino-2-phenylindole (DAPI) (MP Biomedicals) in 1% BSA in PBS for 30 minutes. Finally, the gels were washed six times with 1 % BSA in PBS in preparation for visualization with microscopy.

2.9 Microscopy

Spheroids were visualized using an IX-71 Olympus microscope under brightfield/relief contrast and 100X magnification. DAPI (i.e., nuclear) staining was visualized using ultraviolet/fluorescence illumination. Brightfield and fluorescence images of spheroids were acquired using a Hammamatsu camera interfaced to the microscope using SimplePCI Imaging software on a PC computer. Images of at least 6 different spheroids were acquired for each experimental condition tested. Moreover, two different focal planes were captured for each spheroid to aid in recognition of sprout-like structures.

2.10 Morphometric Analyses

The tubulogenic activity of 3-D endothelial spheroid cultures were assessed by quantifying their formation of sprout-like structures that extended from the end of the main body of the spheroid. Sprouts were identified as distinguishable linear structures with at least two nuclei and/or > 50 μm in projected length on the imaged focal plane of the spheroid. Sprouts were quantified by manual counts (number of sprouts/spheroid), cumulative projected length of sprouts/spheroid, and average projected length of sprouts/spheroid using ImageJ software.

Figure 2.4 shows an example of a how a spheroid was quantified morphometrically. Panel i and panel ii depict identical spheroids that would be captured under bright field illumination for imaging morphology and fluorescence for visualizing DAPI-stained nuclei, respectively. By comparing those two fields, we identified sprouts to be tube-like structures that contained at least two nuclei and/or were at minimum 50 μm in length (Panel iii). When branched structures were observed, the total length of all the branches for that sprout originating from the spheroid was measured as one sprout.

2.11 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). The measured tubulogenic activities of spheroid cultures were assessed using raw values. Comparison between means of experimental treatments were conducted using paired Student's t-test with $p < 0.05$ denoting significant differences. To test the effects of MAZ51, we first normalized the tubulogenic activity of pressurized cells to that of controls and used this

parameter to determine the fold change caused by pressure stimulation. Significant fold changes were determined using one-sample t-tests that compared experimental values to a reference value of 1.

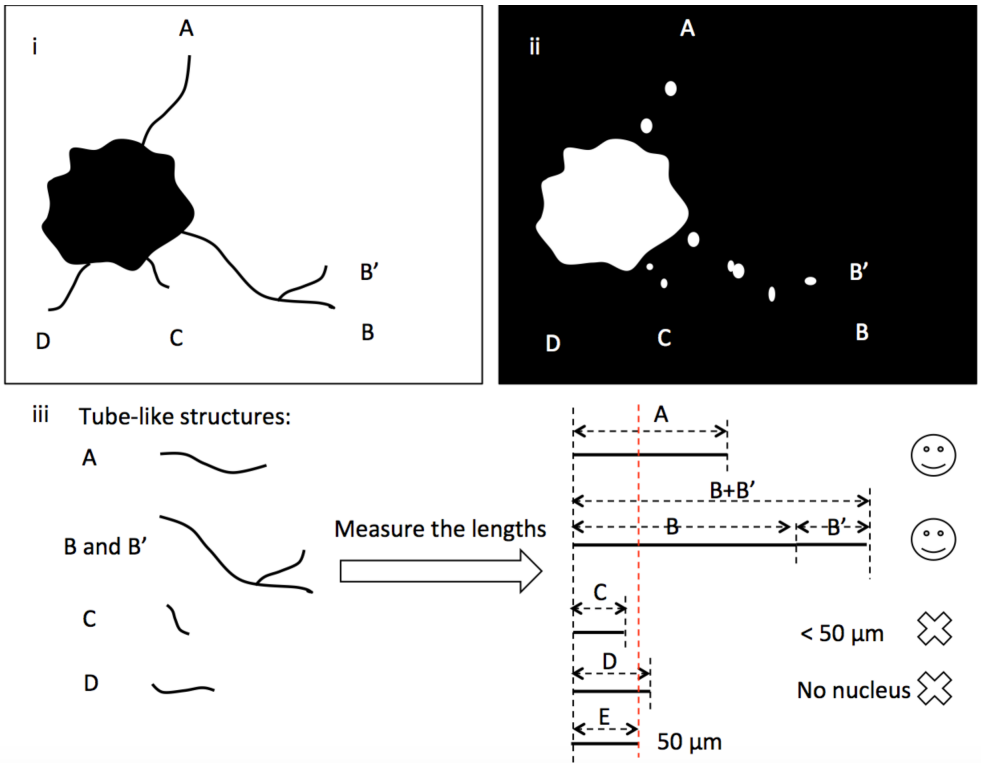

Figure 2.4. Quantifying the Sprouting Activity of Endothelial Spheroids in 3-D Collagen Gels. Spheroids will be analyzed based on their brightfield (i) and UV fluorescence (ii) images. In this example, A, B (and B'), C, and D are identified to be sprout candidates that were quantified (iii). In this example provided, only structures A and B+B' would be included in our analyses based on their nuclear content (>2) and length (>50 µm).

3. Results

3.1 Distribution of tube-like structures with two nuclei

We conducted an analysis to verify that sprouts of at least 50 µm in length satisfied the criterion that they contained at least 2 nuclei. Histogram analyses (Figure 3.1) of the lengths of 298 tube-like structures that were measured from spheroids cultured under control (i.e., atmospheric) pressure conditions for 2 days revealed that at least 96.3% are longer than 50 µm (Figure 3.1). On average, the length of sprout-like structures for controls was 109.89 µm ± 2.15 µm (mean ± SEM). Finally, sprout lengths among the population analyzed followed a normal distribution.

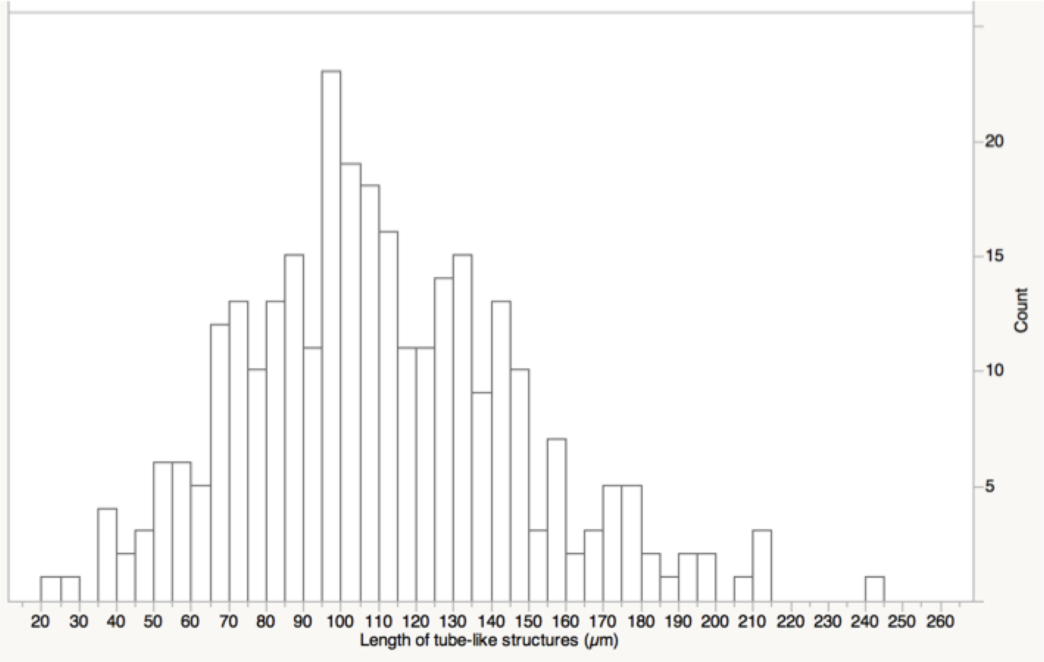

Figure 3.1. Histogram of sprout lengths of all the spheroids cultured for 2 days that have at least two nuclei.

3.2 BAEC spheroids enhanced sprouting after exposure to 20 mmHg for 3 days

BAEC spheroids suspended in collagen gels exhibited sprout formation when maintained under control (atmospheric) pressure conditions (Figure 3.2, Panel A) or exposed to 20 mmHg hydrostatic pressures (Figure 3.2, Panel B) for 3 days.

The spheroids exposed to 20 mmHg for 3 days exhibited significant increases in cumulative length of sprouts/spheroid and average length of sprouts/spheroid relative to the controls (Figure 3.3). In contrast, in these experiments, we did not detect any changes in number of sprouts/spheroid in BAECs exposed to 20 mmHg (Figure 3.3).

3.3 Endothelial sprouting exhibits a complex pressure magnitude dependence after 3 days

BAEC spheroids suspended in collagen gels exhibited sprout formation when maintained under control (atmospheric) pressure conditions or exposed to 5mmHg, 20 mmHg, or 40 mmHg hydrostatic pressures (Figure 3.4) for 3 days. After exposure to 5 mmHg for 3 days, BAECs exhibited significant decreases in cumulative length of sprouts/spheroid and average length of sprouts/spheroid relative to the controls (Figure 3.5). There were no differences detected in number of sprouts/spheroid for BAECs either maintained under control conditions or exposed to 5 mmHg (Figure 3.5). The spheroids exposed to 20 mmHg for 3 days exhibited significant increases in number of sprouts/spheroid, cumulative length of sprouts/spheroid and average length of sprouts/spheroid relative to the controls (Figure 3.5). Finally, spheroids exposed to 40 mmHg for 3 days exhibited significant decreases in the number of sprouts/spheroid

relative to controls (Figure 3.5). But cumulative length of sprouts/spheroid and average length of sprouts/spheroid did not show significant changes due to 40 mmHg-pressure exposure (Figure 3.5).

3.4 Pressure-sensitive endothelial sprouting is duration dependent

BAEC spheroids suspended in collagen gels exhibited sprout formation when maintained under control (atmospheric) pressure conditions or exposed to 5mmHg (Figure 3.6), or 20 mmHg (Figure 3.7), or 40 mmHg (Figure 3.8) hydrostatic pressures for 2 days. Spheroids exposed to 5 mmHg for 2 days exhibited significant decreases in number of sprouts/spheroid, cumulative length of sprouts/spheroid and average length of sprouts/spheroid relative to the controls (Figure 3.9). The spheroids exposed to 20 mmHg for 2 days did not show significant difference in any of the measures of sprout activity compared to controls (Figure 3.9). Finally, spheroids exposed to 40 mmHg for 2 days exhibited significant decreases in average length of sprouts/spheroid relative to the controls. But number of sprouts/spheroid and the cumulative length of sprouts/spheroid were unaffected by exposure to 40 mmHg for 2 days (Figure 3.9).

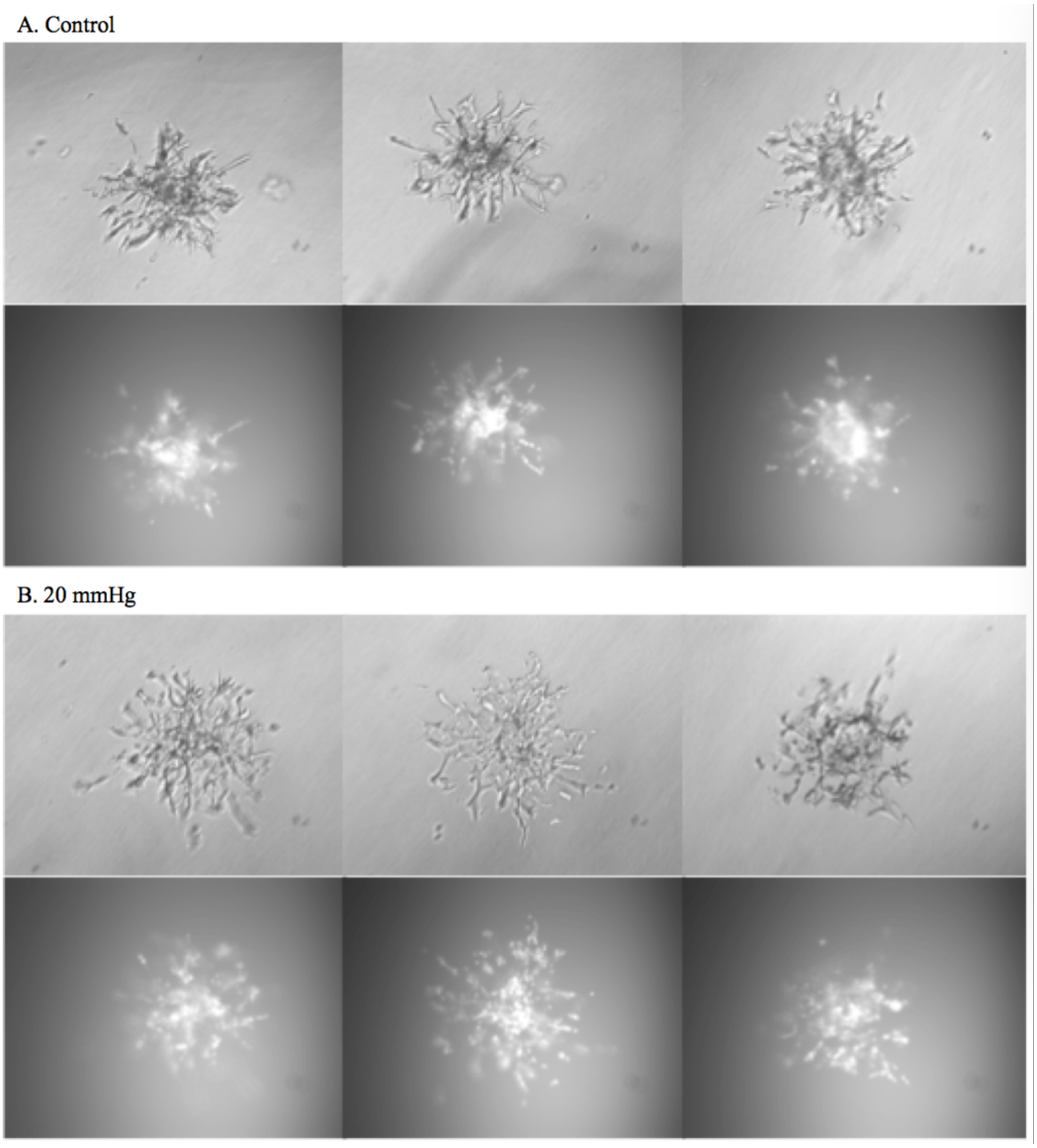

Figure 3.2. Representative images of BAEC spheroids exposed to 20 mmHg for 3 days. Panel A: The brightfield and DAPI fluorescence images depict BAEC spheroids cultured maintained under control (atmospheric pressure) conditions for 3 days. Panel B: Brightfield and DAPI fluorescence images of BAEC spheroids exposed to 20 mmHg hydrostatic pressures for 3 days. DAPI fluorescence images displayed nuclear distributions within the sprouts and in the spheroid body. Magnification is 100X.

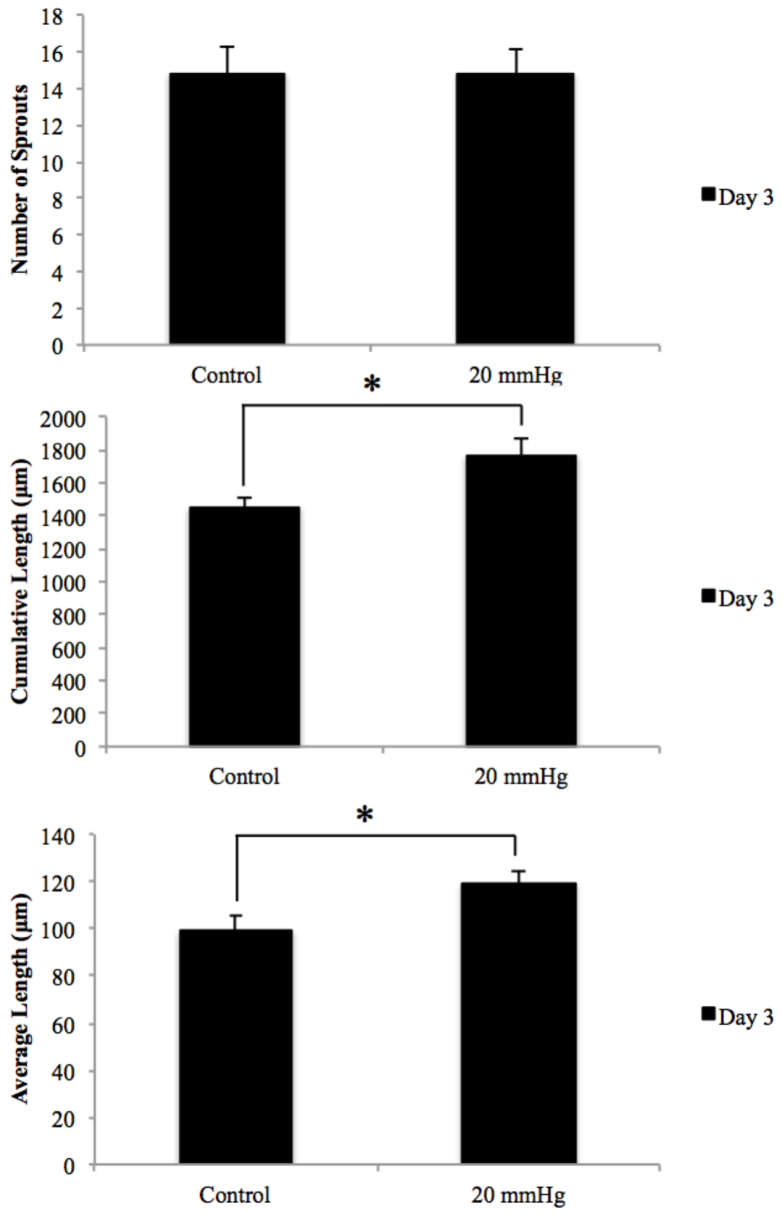

Figure 3.3. Endothelial spheroids exposed to 20 mmHg for 3 days appears to enhance endothelial sprouting. Bars are mean ± standard error; n = 3 independent experiments. *p < 0.05 compared to match controls using paired Student's t-test.

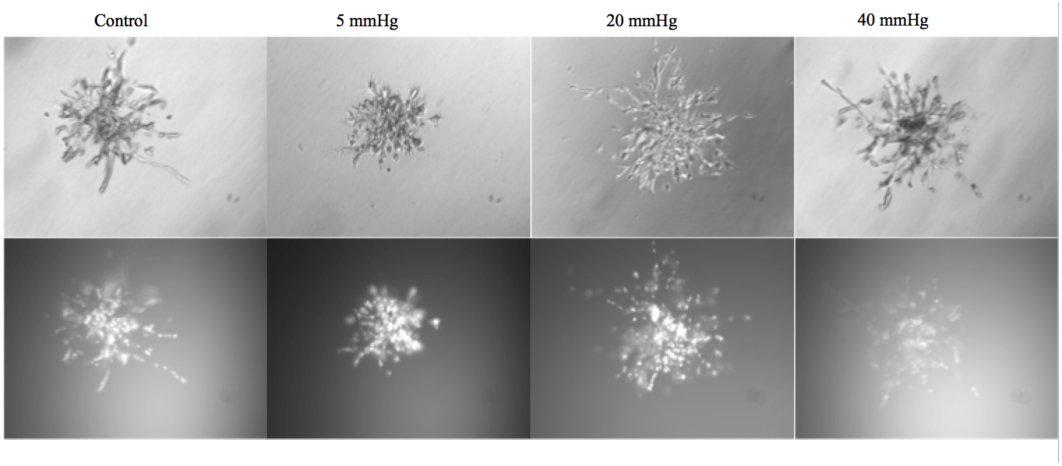

Figure 3.4. Representative images of BAEC spheroids exposed to different pressure levels for 3 days. The brightfield and DAPI fluorescence images of BAEC spheroids suspended in collagen gels either maintained under control (atmospheric) pressure conditions or exposed to either 5 mmHg or 20 mmHg or 40 mmHg hydrostatic pressures for 3 days. DAPI fluorescence images displayed nuclear distributions within the sprouts and in the spheroid body. Magnification is 100X.

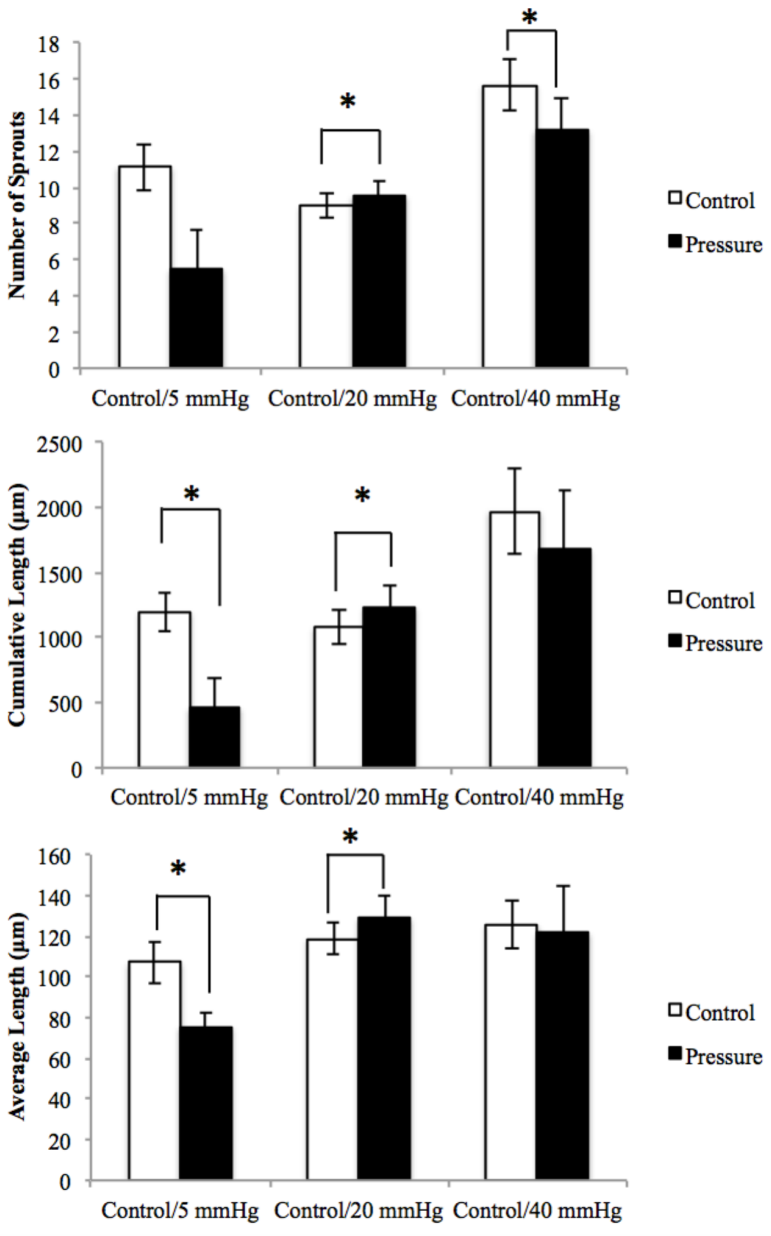

Figure 3.5. Endothelial spheroids exposed to 5, 20, and 40 mmHg for 3 days shows a complex effect on endothelial sprouting. Bars are mean ± standard error; n = 3, 4 independent experiments. *p < 0.05 compared to match atmospheric pressure controls using paired Student's t-test.

3.5 Pressure-induced BAEC spheroid sprouting is VEGFR-3 dependent

Pressurized and unpressurized BAEC spheroids suspended in collagen gels exhibited sprouting activity in the absence (Figure 3.10) and presence of either 0.5 μM MAZ 51 (Figure 3.11) or 0.01% DMSO (Figure 3.12) for 3 days. While untreated BAEC spheroids exposed to 20 mmHg for 3 days exhibited significant increases in the number of sprouts/spheroid, cumulative length of sprouts/spheroid and average length of sprouts/spheroid relative to the controls (Figure 3.13), those exposed to similar pressures but in the presence of 0.5 μM MAZ51 only exhibited significant increases of average length of sprouts/spheroid, but not the number of sprouts/spheroid or cumulative length of sprouts/spheroid (Figure 3.13). Finally, spheroids exposed to 20 mmHg with vehicle control (i.e., 0.01% DMSO) for 3 days exhibited significant increases of cumulative length of sprouts/spheroid and average length of sprouts/spheroid, but not number of sprouts/spheroid relative to similar spheroid preparations that had been maintained under control pressures, (Figure 3.13). Together, these data implicated VEGFR-3 involvement in the sprouting responses of BAECs to 20-mmHg pressure exposure.

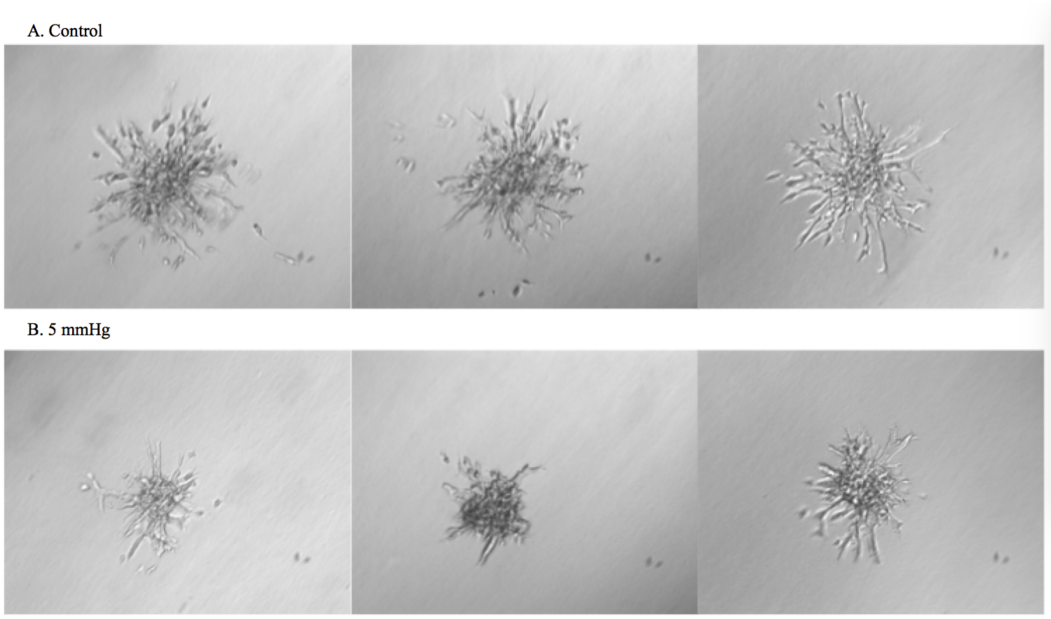

Figure 3.6. Representative images of BAEC spheroids exposed to 5 mmHg for 2 days. Panel A: The brightfield images depict BAEC spheroids cultured under atmospheric conditions for 2 days. Panel B: The brightfield images depict BAEC spheroids exposed to 5 mmHg hydrostatic pressures for 2 days. Magnification is 100X magnification.

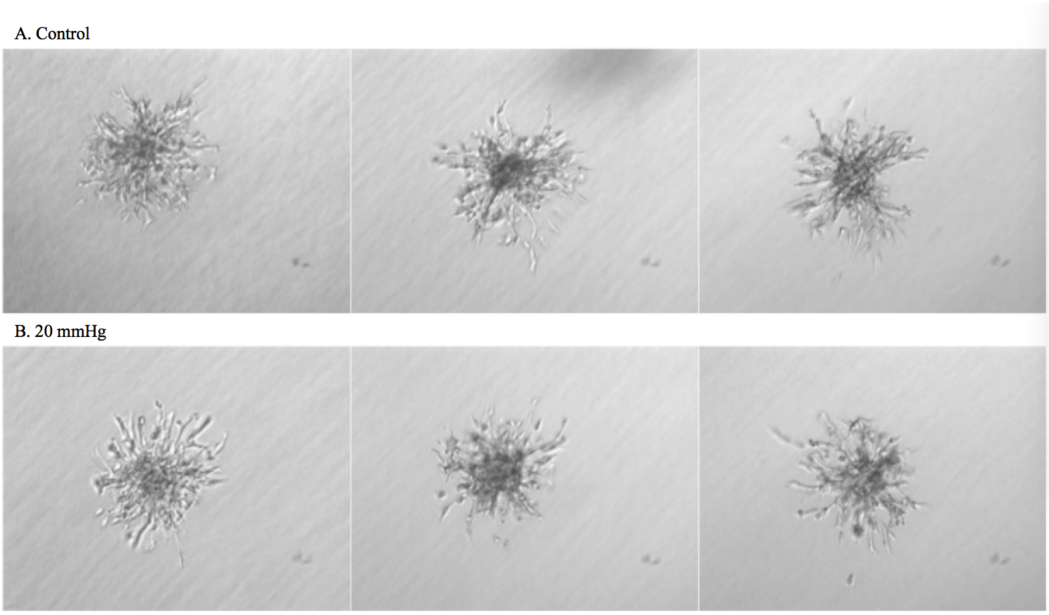

Figure 3.7. Representative images of BAEC spheroids exposed to 20 mmHg for 2 days. Panel A: The brightfield images depict BAEC spheroids cultured under atmospheric conditions for 2 days. Panel B: The brightfield images depict BAEC spheroids exposed to 20 mmHg hydrostatic pressures for 2 days. Magnification is 100X magnification.

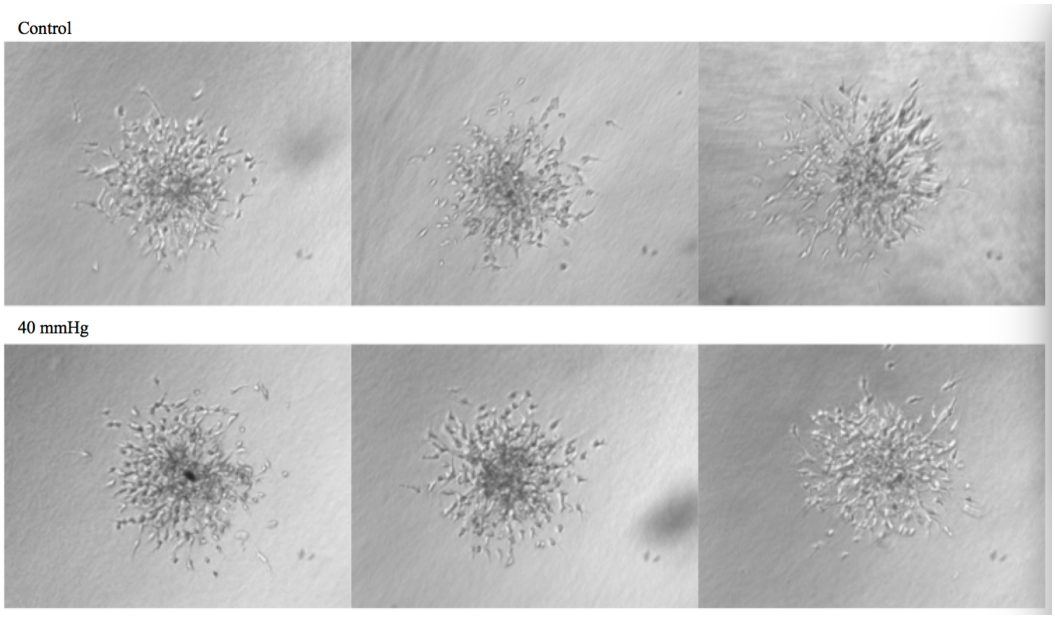

Figure 3.8. Representative images of BAEC spheroids exposed to 40 mmHg for 2 days. Panel A: The brightfield images depict BAEC spheroids cultured under atmospheric conditions for 2 days. Panel B: The brightfield images depict BAEC spheroids exposed to 40 mmHg hydrostatic pressures for 2 days. Magnification is 100X magnification.

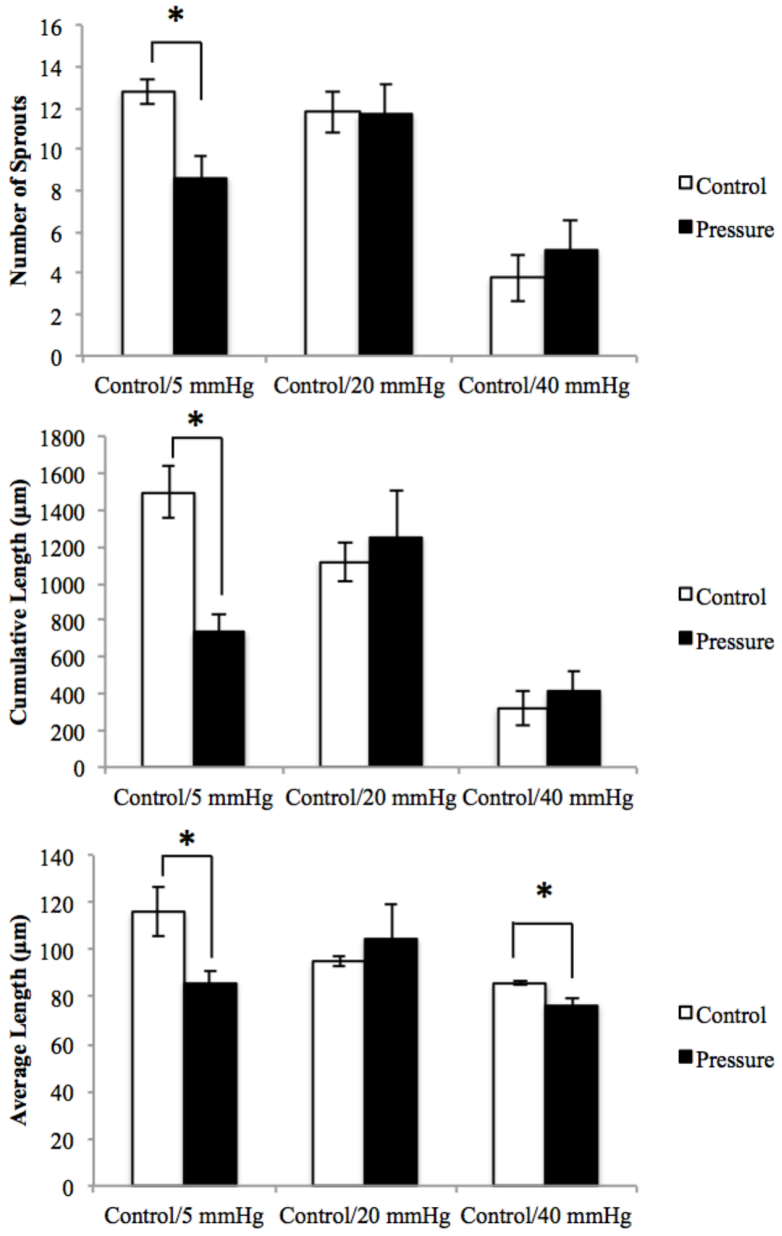

Figure 3.9. Endothelial spheroids exposed to 5, 20, and 40 mmHg for 2 days shows a complex effect on endothelial sprouting. Bars are mean ± standard error; n = 3 independent experiments. *p < 0.05 compared to match controls using paired Student's t-test.

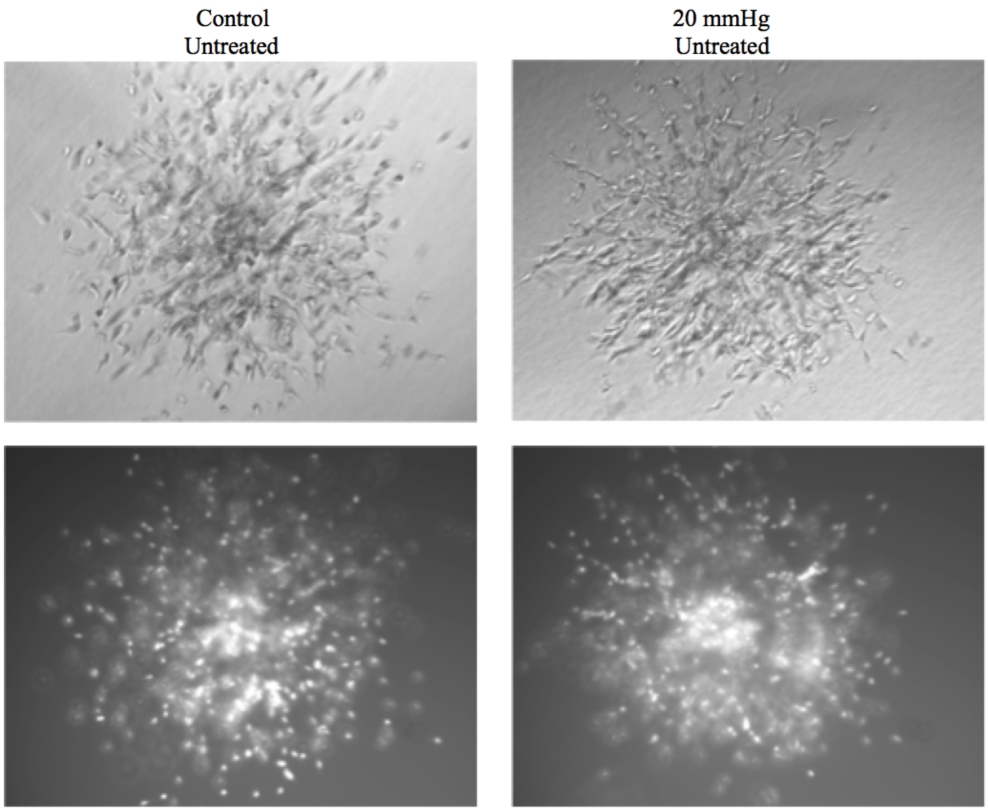

Figure 3.10. Representative images of BAEC spheroids exposed to pressure for 3 days. The brightfield and fluorescence images depict BAEC spheroids suspended in collagen gels and either maintained under control (atmospheric) pressure conditions or exposed to 20 mmHg hydrostatic pressures for 3 days. DAPI fluorescence images displayed nuclear distributions within the sprouts and in the spheroid body. Magnification is 100X magnification.

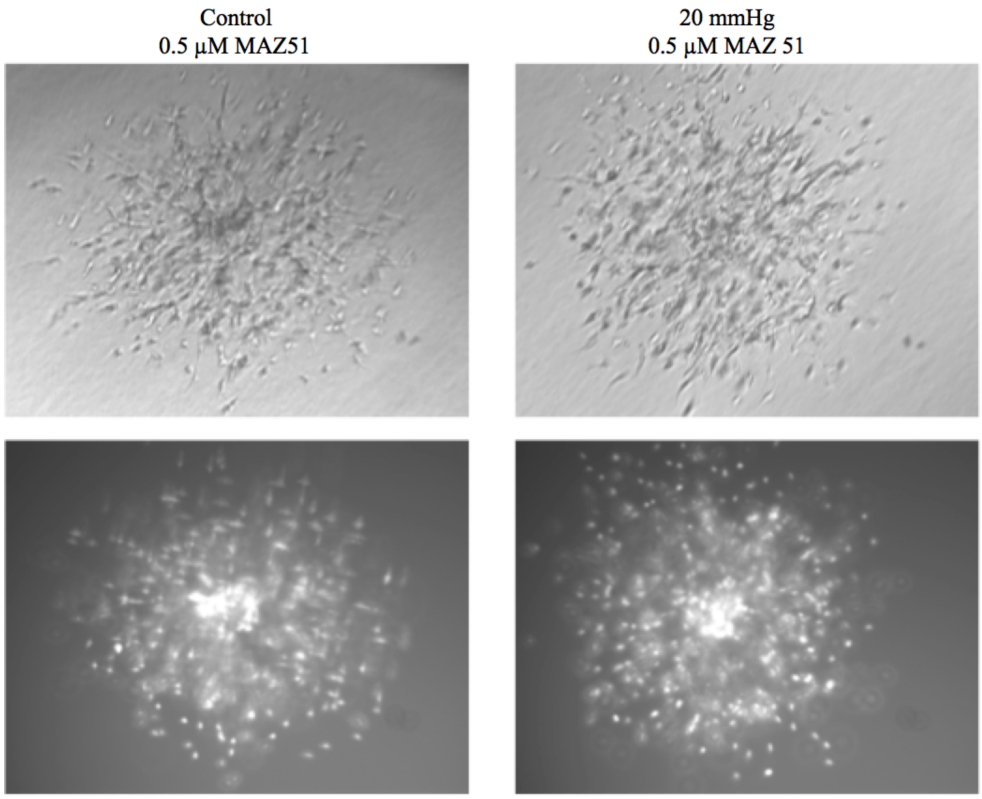

Figure 3.11. Representative images of BAEC spheroids exposed to hydrostatic pressure in the presence of 0.5 µM MAZ 51 for 3 days. The brightfield and fluorescence images depict BAEC spheroids suspended in collagen gels and either maintained under control (atmospheric) pressure conditions or exposed to 20 mmHg hydrostatic pressures for 3 days. DAPI fluorescence images displayed nuclear distributions within the sprouts and in the spheroid body. Magnification is 100X magnification.

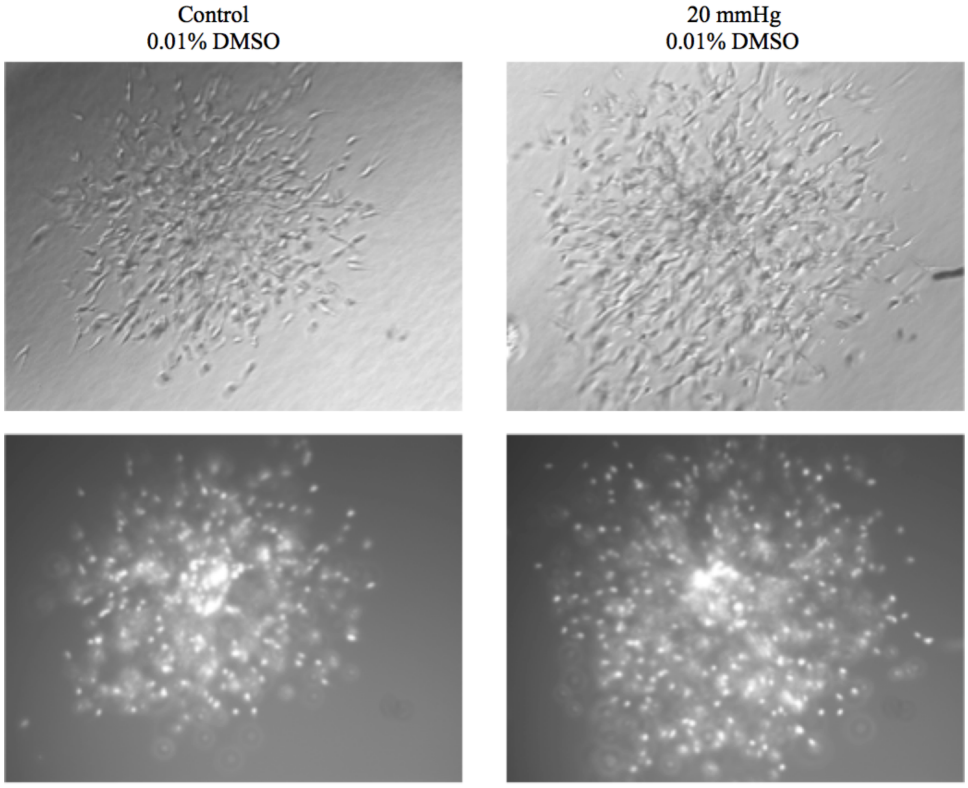

Figure 3.12. Representative images of BAEC spheroids exposed to hydrostatic pressure in the presence of DMSO cultured for 3 days. The brightfield and fluorescence images depict BAEC spheroids suspended in collagen gels and either maintained under control (atmospheric) pressure conditions or exposed to 20 mmHg hydrostatic pressures for 3 days. DAPI fluorescence images displayed nuclear distributions within the sprouts and in the spheroid body. Magnification is 100X magnification.

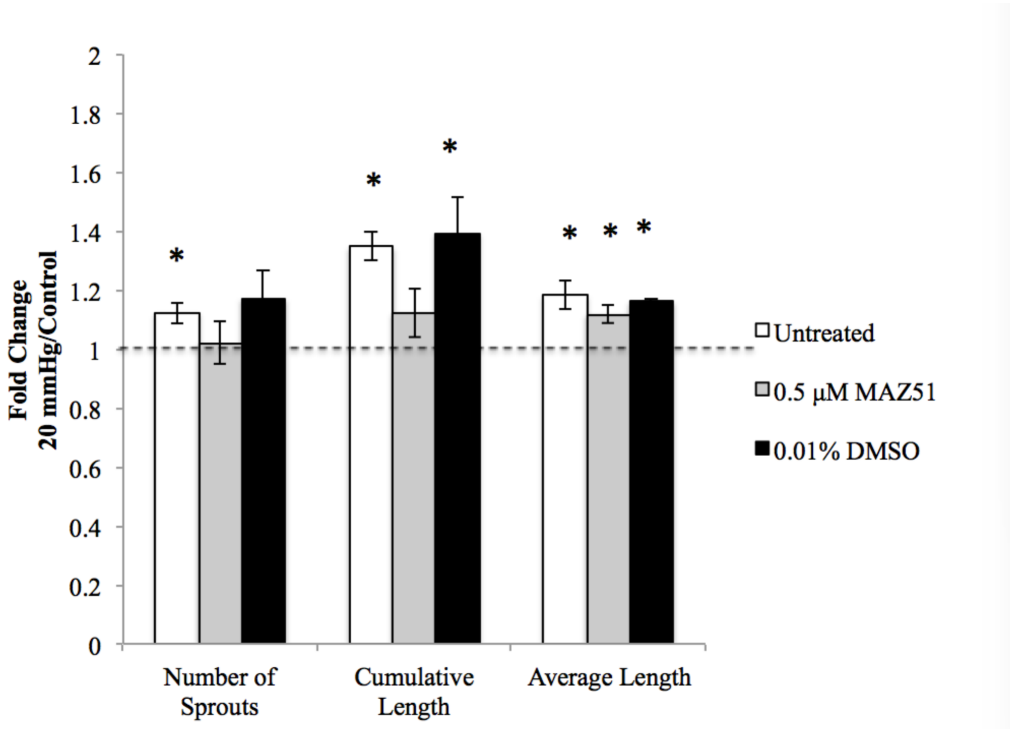

Figure 3.13. Expression of VEGFR-3 affects endothelial tubulogenic activities in the presence of pressure. Bars are mean ± standard error; n = 3. *p < 0.05 compared to match controls using paired Student's t-test.

4. Discussion

The present study tested the hypothesis that hydrostatic pressure stimulates endothelial sprout formation in a magnitude and exposure time dependent fashion involving VEGF-C/VEGFR-3 signaling. To address this hypothesis, the study was designed with two aims. For the first aim, we conducted 3-D collagen gel assays to characterize the pressure sensitivity of in vitro endothelial tubulogenesis by BAECs in the range of 0 (atmospheric) to 40 mmHg for periods of time up to 3 days. In the second aim, we used a small-molecule inhibitor, MAZ51, to explore the involvement of VEGFR-3 in pressure-induced tubulogenesis. VEGFR-3 and its high affinity ligand, VEGF-C, has been shown to be upregulated by hydrostatic pressure (Shin et al., 2012) and is considered to be involved in lymphangiogenesis (Hoeben et al., 2004).

4.1 Physiological conditions of hydrostatic pressure

In the circulatory system, endothelial cells are exposed to different pressures depending on their locations in the vessels and the physiological conditions of surrounding environment. During angiogenesis and lymphangiogenesis, endothelial cells may also experience interstitial pressures as they migrate into and invade the extravascular space. In this study we are interested in tubulogenic processes (i.e., angiogenesis or lymphangiogenesis) that occur in the microcirculation and interstitial tissues, including the lymphatics.

The three pressure levels tested in this study to assess the pressure sensitivity of endothelial tubulogenic activity were 5 mmHg, 20 mmHg, and 40 mmHg sustained hydrostatic pressures. Because tubulogenic processes start in microvasculature and develop into the surrounding tissues, the 5 mmHg and 20 mmHg pressure levels used in this study were chosen to fall within the physiological range of 0 to 30 mmHg typical of the microcirculation and of -8 mmHg to 20 mmHg reported for the interstitium (Guyton Arthur & Hall Jhon, 1996). The 40 mmHg hydrostatic pressure was chosen as a "high," potentially pathological stimulus since it is higher than what would be expected under normal physiological conditions in the capillaries, venules, or tissues where endothelial cells carry out their tubulogenic activity. For example, under disease conditions such as in the hypertensive pulmonary artery, pressures can reach 35 mmHg to 40 mmHg (Tuder & Voelkel, 2002). Interstitial fluid pressures can rise above 50 mmHg in tumors (Boucher & Jain, 1992).

There is previous evidence that pressures within this range affect tubulogenic activity. Briefly, Acevedo et al. showed that 1.5 – 15 cm H_2O (approximately 1.1 mmHg – 11 mmHg) stimulates bovine pulmonary artery endothelial cell elongation, proliferation, and release of FGF-2 (Acevedo et al., 1993). Exposure to 20 mmHg has been reported to upregulate VEGF-C and VEGFR-3, and promote endothelial proliferation and sprout formation (Shin et al., 2012). Furthermore, it has been shown that FGF-2 is involved in the regulation of endothelial cells to pressure stimuli (Acevedo et al., 1993; Schwartz et al., 1999; Shin, Schwartz, Bizios, & Gerritsen, 2004; Shin et al., 2012). Based on evidence like these, we rationalized the use of 5, 20, and 40 mmHg to examine the pressure and exposure time dependence of endothelial tubulogenic activity.

4.2 Cell selection

BAECs are capable of forming tube-like structures in vitro (Dietrich & Lelkes, 2006) and have been used in many investigations regarding the effects of pressure on endothelial tubulogenic processes such as proliferation and expression of angiogenic molecules (Ohashi, Sugaya, Sakamoto, & Sato, 2007). For example, BAEC morphology and proliferation rates are altered by static pressure elevations in a magnitude and duration dependent fashion (Sumpio et al., 1994). Moreover, BAECs express both VEGF-C (Kumar, Harris-Hooker, & Sanford, 2008) and VEGFR-3 (Persaud et al., 2004). Thus, it was appropriate for us to examine the role of VEGF-C/VEGFR-3 signaling in pressure-induced tubulogenesis. In the present study, we observed BAEC spheroids to be capable of forming tube-like structures (see section 3.2).

4.3 3-D gel assay

The use of 3-D models to assess endothelial tubulogenic activity was selected to more accurately mimic the in vivo environment for endothelial cells. It not only allowed us to study endothelial sprout formation as a whole but also provided some insight regarding the relative contributions of pressure-sensitive proliferation and migration, two key processes that play key roles in tubulogenesis.

Hydrogels, similar to those used in the present study, are made of complex cross-linked polymer chains or protein molecules. They are typically employed as biological

matrices to mimic ECM because of their high water contents, transport of gases, nutrients, wastes, and soluble factors as well as their ability to support the growth of cells in a 3-D geometry (Tibbitt & Anseth, 2009).

Three common hydrogels for 3-D cell cultures are type I collagen gel, Matrigel, and fibrin gel. Native ECM and basement membrane is typically comprised of type I collagen, type IV collagen, and laminin (Carmeliet, 2003; Wozniak & Keely, 2005). Type I collagen is major component of connective tissue. Type IV collagen, and laminin are components of basement membrane.

In this study, we examined the pressure mechanobiology of endothelial tubulogenesis in hydrogel constructs made up of type I collagen. We chose Type I collagen because it is the minimal collagen-based gel component needed to support endothelial growth in 3-D collagenous matrices. Matrigel was avoided since it contains other constituents which may vary widely from batch to batch. Fibrin gel contains fibrinogen and thrombin which play an important role in blood clotting (Janmey, Winer, & Weisel, 2009). So fibrin gels are usually used to model clotted tissues during wound healing processes.

4.4 Parameters we studied

For our analyses, we defined a sprout as a linear structure with at least two nuclei. Furthermore, the sprout had to be greater than 50 μm in path length, which was used as a threshold length by others (Dietrich & Lelkes, 2006). To verify that sprouts having a minimum of two nuclei were at least 50 μm in length, we analyzed 298 tube-like

structures formed by our BAECs maintained under control conditions cultured for 2 days. We found that 96.3% of the sprouts having 2 nuclei were longer than 50 μm in length. This provided the confirmation we needed to proceed with our pressure studies.

In the present study, we used three parameters to assess the tubulogenic activity of endothelial cells: number of sprouts/spheroid, cumulative lengths of sprouts/spheroid, and the average length of sprouts/spheroid. The number of sprouts/spheroid is the average number of sprouts extending from each spheroid. The cumulative length of sprouts/spheroid is the average of the sum of the lengths of all sprouts from one spheroid. Finally, the average length of sprouts/spheroid was the mean length of all sprouts extending from each individual spheroid.

Number of sprouts is measured to generally represent the effect of pressure on initiation of endothelial sprout formation which likely involves invasion and migration of endothelial cells. Cumulative length of sprouts/spheroid and average length of sprouts/spheroid gives us two perspectives of the effect of pressure on endothelial sprout outgrowth. The cumulative length of sprouts/spheroid is anticipated to occur due to changes in sprout lengths without direct consideration of number of sprouts. This provided us with an initial assessment of the overall extent of endothelial sprout outgrowth which may be due more so to cell proliferation. We can get a sense of whether the change in cumulative length of sprouts/spheroid is due to the change in number of sprouts (cell invasiveness) or sprout elongation (cell proliferation) using the average length of sprouts/spheroid.

Based on our analyses, we believe that cumulative length of sprouts/spheroid was the most reliable assessment of the tubulogenic activity for endothelial spheroid assay used in the present study. There was a high degree of variability in the numbers of sprouts and branches formed from one endothelial spheroid. It was also challenging at times to identify individual sprouts due to the difficulty in determining the edges of spheroid body from which sprouts extended. As a result, the values for number of sprouts and average length of sprouts/spheroid (which also requires identification of individual sprouts) were associated with a large variability. On the other hand, cumulative length of sprouts/spheroid only relied on measurements that were summed without dependence on identifying individual sprouts, which we defined to originate from the spheroid body. Notably, this is in contrary to the bead assays conducted by Shin et al. (2012). This could be explained by the fact that sprouts extending from the solid, inert surfaces of the microbeads were easier to distinguish.

4.5 Evidence that shows the tubulogenesis is associated with the magnitude and duration of pressure

There is evidence in the literature that tubulogenic activities are modulated by exposure to pressure. Exposure of human umbilical vein endothelial cell (HUVEC) to as low as 3 mmHg is stimulatory for cell proliferation compared to cells cultured under control pressure conditions (Schwartz et al., 1999). Bovine pulmonary artery endothelial cells elongate as well as display increased proliferation rates in response to hydrostatic pressures of 1.5 – 15 cm H_2O for up to 7 days (Acevedo et al., 1993). Acevedo et al. also

showed release of FGF-2 from endothelial cells exposed to pressure. BAECs also elongate and proliferate at higher rates under high pressures (40, 80, and 120 mmHg) depending on exposure times (9, 7, and 3 days respectively) (Sumpio et al., 1994). A previous study also showed an upregulation of VEGF-C and VEGFR-3 when BAECs were exposed to 20 mmHg but not 40 mmHg (Shin et al., 2012). Studies such as these supported the likelihood that hydrostatic pressure is stimulatory for tube formation.

In fact, our laboratory previously showed that sprout formation is stimulated by exposure to 20 mmHg but not to 40 mmHg for 3 days based on a microcarrier bead model of tubulogenesis (Shin et al., 2012). However, the microcarrier bead is non-biodegradable which is questionable to be used in tissue engineering applications. Thus, instead of the microcarrier bead model, we applied endothelial spheroid model in this study to firstly verify if the result is consistent with what was reported from the bead model.

In present study, we explored the hydrostatic pressure magnitude and duration sensitivity of endothelial tubulogenesis by using an endothelial spheroid model. First, we compared the number of sprouts, cumulative length of sprouts/spheroid, and average length of sprouts/spheroid from the spheroids maintained under control conditions and or exposed to 20 mmHg for 3 days. We found significantly increased cumulative length of sprouts/spheroid and average length of sprouts/spheroid but not the number of sprouts for the spheroids exposed to 20 mmHg for 3 days in comparison with controls. However, in a previous study, the investigators did not report any changes in average sprout lengths but they did report an increase in the number of sprouts for BAECs on microcarrier beads exposed to 20 mmHg in collagen gels (Shin et al., 2012). The different results between

the present study and those of Shin et al. (2012) may have resulted from the differences between the two tubulogenesis models used. It is likely that difficulty in distinguishing distinct sprouts originating from spheroid bodies from those that branched from other sprouts may have contributed to a high variability in the measured parameter.

Notably, we consistently showed that exposure of 20 mmHg for 3 days promoted BAEC sprouting as indexed by changes in cumulative length of sprouts/spheroid originating from spheroids. It should be noted that, in our initial experiments (see Section 3.2), we observed this increase in cumulative lengths but not an increase in number of sprouts. However, in our second set of experiments (see Section 3.3), while we did observe an increase in cumulative length of sprouts/spheroid for BAEC spheroids exposed to 20 mmHg, we also observed a significant increase in number of sprouts. The discrepancy in the number of sprouts/spheroid observed for these two sets of experiments supported the likelihood of a high variability in the number of sprouts index used for quantifying BAEC sprouting activity under pressure originating from difficulty identifying individual sprouts based on our definition of what a sprout is. Moreover, the data in the present study confirmed that the cumulative length of sprouts/spheroid measure was a more reliable measure.

The present study also provided evidence that BAEC sprout formation is pressure magnitude dependent in line with prior studies reporting magnitude-dependent effects of pressure on cell proliferation for HUVECs, bovine pulmonary artery endothelial cells, and BAECs. Notably, we found that exposure to 5 mmHg induced an inhibitory effect on BAEC sprout formation while, based on our cumulative length measure, the 40-mmHg pressure had no effect.

Although it has been shown that low pressures (1.5 – 15 cm H_2O) promote bovine pulmonary artery endothelial cell proliferation and elongation (Acevedo et al., 1993), we still observed decreased cumulative length of sprouts/spheroid for BAEC spheroids exposed to 5 mmHg for 3 days. This suggests that low pressure may limit other steps in endothelial tubulogenesis process such as invasion, or migration. More work, however, is needed to explain this discrepancy.

Exposure to 40 mmHg for 3 days had no effect on lengths but it did appear to have an inhibitory effect on number of sprouts/spheroid. BAECs showed increased proliferation when exposed to 40 mmHg only after 9 days (Sumpio et al., 1994). Our laboratory previously showed that exposure to 40 mmHg for 3 days had no effect on BAEC proliferation under the conditions similar to those tested in the present study (Shin et al., 2012). These results indicate that a 3-day exposure to 40 mmHg may not have been enough to induce a change in BAEC sprout formation considering that cumulative length of sprouts/spheroid result endothelial cell proliferation during sprout elongation (Shin et al., 2012). Notably, we did detect a significant decrease in number of sprouts/spheroid, which is different from previous observations (Shin et al., 2012), but again this difference may have been due to the different models or the variability associated with this parameter assessing sprouting activity of BAEC spheroids.

Finally, we examined the dependence of pressure-sensitive BAEC sprouting on exposure time. We found that 5 mmHg showed an inhibitory effect on number of sprouts/spheroid and cumulative length of sprouts/spheroid suggesting that the inhibitory effects of the 5-mmHg stimulus occurs rapidly within 2 days of the onset of pressure stimulation. In the case of the 20-mmHg stimulus, enhanced BAEC sprout formation

required at least 3 days of pressure exposure. Notably, BAEC spheroids exposed to 40 mmHg showed only a decrease in average length of sprouts/spheroid but not number of sprouts/spheroid and cumulative length of sprouts/spheroid. Taken together, these data indicates that 2-day exposure times are enough for endothelial spheroids to show reduced sprouting activity (e.g., when exposed to 5 mmHg) while 3-day exposure times are required for BAEC spheroids to display enhanced sprout formation (e.g., in response to 20 mmHg). Thus, BAEC sprouting is pressure exposure duration dependent. Moreover, the regulation of endothelial tubulogenic activity is complex that may involve different mechanisms depending on the applied magnitude of the stimulus.

4.6 Tubulogenic activities are VEGFR-3 dependent

In addition to characterizing the pressure dependence of endothelial sprouting activity, the present study also aimed to determine if VEGFR-3 is involved in pressure-induced tubulogenic activities. BAECs express both VEGF-C (Kumar et al., 2008) and its receptor VEGFR-3 (Persaud et al., 2004). Although the VEGFR-3 is widely thought to be involved in lymphangiogenesis and associated with lymphatic endothelial cells, VEGFR-3 has been reported to be expressed by blood vessel endothelial cells including BAEC (Pepper et al., 1998; Persaud et al., 2004). So we cannot exclude the possibility that the pressure-induced tubulogenesis is angiogenic even if VEGFR-3 is involved.

Previous studies showed pressure-sensitive expression of VEGF-C (Shin et al., 2002; Shin et al., 2012). It has also been reported that expression of VEGF-C and the HUVEC proliferation is increased for HUVEC exposed to 60/20 mmHg cyclic pressure.

Recently, it was reported that VEGF-C and VEGFR-3 expression is upregulated by exposure of BAEC to 20 mmHg, but not 40 mmHg (Shin et al., 2012). Considering that VEGFR-3 was also reported to play a role in the upregulation of BAEC proliferation by exposure to 20 mmHg, it is conceivable that VEGFR-3 is also involved in the enhanced sprouting activity of BAECs exposed to 20 mmHg. Using the small molecule inhibitor, MAZ51, which blocks the kinase activity of this receptor (Kirkin et al., 2004), the results of the present study supported a role for VEGR-3 in pressure-sensitive tubulogenic activity of BAECs. Further evidence is needed to fully reveal how VEGFR-3 participates in this process.

4.7 Summary of Findings

The results of the present study provided evidence for the following:

- BAEC spheroids require at least 3 days of exposure to 20 mmHg to exhibit enhanced sprouting activity in the form of increased sprout length.
- Exposure to 5 mmHg appears to attenuate endothelial sprouting activity.
- BAEC spheroids exposed to 40 mmHg for 3 days also inhibits sprouting activity by reducing sprout numbers, but not lengths.
- Pressure-sensitive VEGFR-3 activity is required for the ability of 20-mmHg pressure stimulus to enhance BAEC sprouting.

Based on these findings and those from a previous study (Shin et al., 2012), it is apparent that VEGFR-3 dependent BAEC sprouting appears to exhibit a complex pressure dependence. These outcomes not only provide new mechanistic insight related to a role for pressure-sensitive tubulogenic regulation in diseases associated with interstitial hypertension, but the also point to a level of control that may be useful for tissue engineering approaches to microvascularize synthetic constructs.

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- Song, M., Wallin, J., and Shin, H.Y. (**2016**), An in Vitro Investigation of Tubulogenic Sprout Formation by Endothelial Cells under Pressure. (Poster) 11th Annual CCTS Spring Conference, Lexington, KY.
- Song, M., Wallin, J., and Shin, H.Y. (**2015**), Exploring Hydrostatic Pressure as a Mechanobiological Stimulus of Endothelial Sprouting. Oral presentation at Biomedical Engineering Society (BMES) 2015 Annual Meeting, Tampa, FL.
- Song, M., Wallin, J., and Shin, H.Y. (**2015**), Characterization of the Dependence Endothelial Sprout Formation Rates on Hydrostatic Pressure. (Poster) 18th Annual Gill Heart Institute Cardiovascular Research Day, Lexington, KY.

- Song, M., Wallin, J., and Shin, H.Y. (**2015**), An Investigation to Resolve the Dependence of Endothelial Sprouting Rates on Hydrostatic Pressure Levels in the Cellular Microenvironment. (Poster) 10th Annual CCTS Spring Conference, Lexington, KY.
- Song, M., Wallin, J., and Shin, H.Y. (**2015**), Ongoing Study to Characterize the Dependence Endothelial Sprout Formation Rates on Hydrostatic Pressure. (Poster) Biomaterials Day, Nashville, TN.

Publication

- Qin, Y., Chen, F., Song, M. (**2012**), On the Synthesis and Characterization of Compound 7-(piperidin-4-yl)-lH-benzo[d][1,3]oxazin-2(4H)-Ketone, ***Journal of Wuhan Institute of Technology***, 11(3): pp. 91-94.