Low Oxygen Treatment for the Optimization of 3D Endothelial Cell Tissue Cultures

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Abstract

Currently, the most salient challenge in tissue engineering is the inability to sufficiently vascularize engineered tissues. Without adequate vasculature, most tissues cannot survive due to lack of nutrient and oxygen diffusion. Thus, the therapeutic capability of tissue engineering remains limited.

A number of studies have shown that mimicking in *vivo* oxygen tensions (4%-6%O_2) promotes proliferation and improves cellular organization of endothelial cells in 2D *in vitro* cultures (Zhao et al. 2008, Decaris et al. 2009, Zhou et al. 2000). Moreover, one recent study has shown that 5% oxygen pre-treatment increases endothelial cell retention on PET scaffolds under dynamic culture, illustrating the potential of low oxygen culture for engineering vascular tissue grafts (Zhao et al. 2008). In this study, we attempted to engineer a pre-vascularized tissue culture by optimizing oxygen concentration. Through the use of low oxygen (5% O_2) environments we aimed to (1) stimulate proliferation and cellular organization of endothelial cell tissue cultures on a 3D porous silk scaffold, and (2) improve cellular retention on 3D silk scaffolds in dynamic cultures. We assessed the potential of low oxygen pre-culture through a series of methods: DNA quantification assays, which were used to monitor cell proliferation; histology and confocal microscopy, which were used to assess cell organization; and real time RT-PCR, which assessed the cells' synthesis of extra cellular matrix (ECM) adhesion proteins, such as CD31, in response to low oxygen.

While preliminary DNA quantification results suggested that a 5% oxygen environment improved endothelial cell proliferation compared to 21% oxygen controls, subsequent studies yielded conflicting results. Studies also showed that low oxygen did not significantly affect cell retention in dynamic tissue cultures, and it did not affect CD31

transcript levels, as compared to 21% oxygen controls. Finally, analysis by histology and confocal microscopy did not show improved endothelial organization between oxygen groups.

Acknowledgements

First and foremost, I would like to thank Leah Bellas for being my mentor over the past two years. Her guidance, patience, and support, as well as her help with countless lab techniques, made this project possible. I thank Kate Blanton for training me on quantitative RT-PCR and Martin Hunter for training me on the Leica microscope and histology equipment. I would also like to thank Balaji Subramanian and Wei-Che Ko for Western blot training as well as for general guidance with my project.

I thank professor Kaplan for letting me work on this project and for being my mentor and advisor. His mentorship and support was a tremendous asset over the past two years.

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List of Abbreviations

Ang-2 – angiopoietin-2
bFGF – basic fibroblast growth factor
ECs – endothelial cells
ECM – extra cellular matrix
EGF – epidermal growth factor
EPCs – endothelial progenitor cells
FIH-1 – factor inhibiting HIF-1
hASCs – human adipose derived stem cells
HFIP – hexafluoroisopropanol
HIF – hypoxia inducible factor
HREs – hypoxia responsive elements
HUVECs – human umbilical vein endothelial cells
HMVECs – human microvascular endothelial cells
HSCs – hematopoietic stem cells
IGF – insulin-like growth factor
MMPs – upregulate matrix metalloproteases
PECAM-1/CD31 – platelet endothelial cell adhesion molecule
PHDs – hydroxylase domain proteins
SMCs – smooth muscle cells
VEGF – vascular endothelial growth factor

Chapter 1: Introduction

The current need for tissue transplants vastly outnumbers the supply of donor tissue. Moreover, immuno-rejection remains a significant problem in allograft transplantation. Regenerative medicine, which aims to restore function of diseased tissue through implants of engineered tissue, is a promising solution to these problems.

While there has been success in the engineering of a few tissues, these successes have been limited to avascular tissues (such as cartilage), or thin tissues with slow metabolism (such as skin and bladder tissues), which need a limited supply of nutrients or can be quickly vascularized by the host (Rivron et al. 2008). The design of most other tissues requires sufficient vascularization to provide nutrients to and remove wastes from cells. Indeed, inadequate vascular structure in implanted tissue often leads to cell death due to insufficient oxygenation and nutrition. Thus, it is no surprise that *in vitro* angiogenesis was identified as the strategically most important category of current tissue engineering research in a 2007 survey about strategic directions in tissue engineering conducted by the editors of Tissue Engineering (Johnson et al. 2007). While there have been some successes in vascularizing tissues, much work needs to be done to create clinically feasible vascularized tissues.

The goal of this project is to mimic *in vivo* oxygen conditions to promote neovascularization of a 3D tissue engineered construct. The specific aims are to (1) promote proliferation and organization of an endothelial cell (EC) tissue culture in a 3D silk scaffold by mimicking *in vivo* oxygen tensions (~5% O_2); and (2) to improve cellular retention of endothelial cells on the scaffold in dynamic culture by manipulating oxygen tensions.

The long term goal is to (1) use this short-term monoculture as a pre-vascularized platform for tissue engineered co-cultures, and (2) use low oxygen tensions as a means to improve endothelial cell retention in dynamic cultures.

Chapter 2: Background

2.1 Vasculogenesis and Angiogenesis *in vivo*

2.1.1 Vascular physiology

The vascular system is an extensive, highly branched network essential for the transport of liquids and gasses, nutrients and wastes, cells and molecular signals. The vascular system is vital for the survival of most tissues, excluding avascualar tissues (Widmaier et al. 2006). *In vivo*, most cell types will not survive when separated by more than 200 microns from a capillary (Morrison 2009)

While there are many classes of vascular tissues, every vessel of the vascular system has one structural characteristic in common, a monolayer of endothelial cells, which line the inner surface of each vessel forming an interior lumen. This inner lining of endothelial cells is essential to the functionality of the vascular system. Endothelial cells serve as a barrier between the blood and tissues, allowing selected permeability of nutrients, wastes, signals and cells, and they also control blood flow by secreting paracrine agents that act on surrounding cells, such as smooth muscle cells (SMCs). Also, endothelial cells mediate angiogenesis, the development of new vessels from preexisting ones, contribute to the creation and maintenance of the extracellular matrix (ECM), and secrete growth factors in response to damage (Widmaier et al. 2006).

Whereas all vessels have endothelial cells, the cellular make up of different tissues varies. Generally, small vessels such as capillaries are only composed of endothelial cells. Medium sized vessels are surrounded by pericytes and fibroblasts, which secrete extracellular matrix (ECM) proteins, and large vessels are lined with smooth muscle cells (SMCs) as well, which regulate dilation of these vessels (Widmaier et al. 2006, Carmeliet 2003). This study

aims to produce capillary-like microvascular networks by using monocultures of endothelial cells.

2.1.2 Vasculogenesis vs. Angiogenesis

Vasculogenesis and angiogenesis are the two mechanisms of *in vivo* blood vessel formation, which is called neovascularization (Francis et al. 2008). Vasculogenesis is defined as *de novo* formation of blood vessels by differentiation of endothelial progenitor cells (EPCs) into endothelial cells, and generally occurs in embryonic development. However, recent evidence suggests that vasculogenesis mediated by EPCs also occurs in adults (Adams and Alitalo 2009). Angiogenesis is the formation of blood vessels by budding and expansion of existing blood vessels (Francis et al. 2008, Adams and Alitalo 2009). This study aims to engineer a vascularized tissue by employing mechanisms that are reminiscent of both vasculogenesis and angiogenesis. While we aim to create blood vessels *de novo*, as occurs during vasculogenesis, our system uses terminally differentiated endothelial cells, which form blood vessels via angiogenesis *in vivo*.

2.1.3 Angiogenesis in vivo

In vivo angiogenesis is mediated by a complex interplay of endothelial and mural cells (SMCs and pericytes), growth factors, signaling molecules and extracellular matrix (ECM) proteins (Figure 1). The chemical signaling of angiogenesis has been studied extensively (Figure 2). Angiogenesis is stimulated by the signaling of angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), angiopoietin-2 (Ang-2), and many others (Papetti and Herman 2002). Signaling by pro-angiogenic molecules can be

triggered by cell responses to hypoxia (Calvani et al. 2006), from tumors (Senger, 1993), and from inflammatory responses to wounds (Bates and Jones 2003).

Angiogenic factors act on endothelial cells (and other cells) to initiate angiogenesis by stimulating cell proliferation, ECM remodeling, cell migration, and eventually tube formation (Otrock et al. 2007) (Figure 1). Proteins such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) activate endothelial cells, causing them to upregulate matrix metalloproteases (MMPs) (Iwasaka et al. 1996), a family of proteins which degrade the ECM of the matrix of endothelial cells. This MMP-mediated ECM degradation is important for endothelial cell migration (Burbridge et al. 2002, Carmeleit 2003). In addition to matrix remodeling, the growth factors (such as VEGF, FGF and EGF) stimulate endothelial proliferation. Once sufficient proliferation occurs, VEGF and FGF facilitate endothelial migration (Papetti and Herman 2002). Endothelial cells activated by growth factors upregulate integrins, such as $\alpha_v\beta_3$ integrin (Senger et al. 1996), which mediate endothelial cell migration by attachment to the ECM (Leavesly et al. 1993). During angiogenic growth and endothelial cell migration, a VEGF mediated pathway activates certain endothelial cells (called tip cells) which lead the formation of sprouts, or premature new endothelial cell cords without lumens (Gerdhard et al. 2003). Recent research suggests that these sprouts form large vacuoles through pinocytosis ("cell drinking"), which eventually fuse with the membrane, forming a lumen (Davis and Bayless 2003, Kemai et al. 2006). Maturation of the newly formed vasculature occurs by recruitment of mural cells and with the synthesis of ECM proteins by endothelial cells and mural cells (Eble and Niland 2009). This recruitment of mural cells is important for the stability and proper function of the vasculature (Jain 2003).

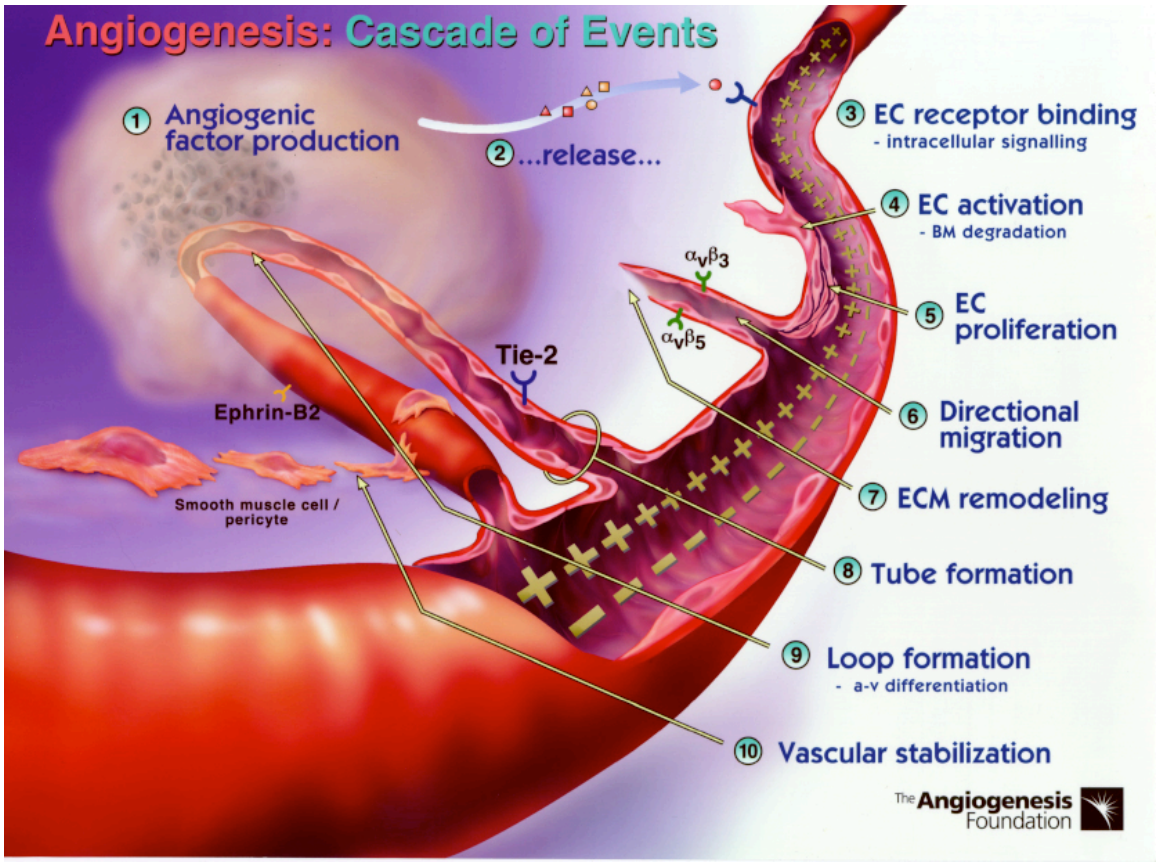

Figure 1:.A schematic diagram of the events that mediate angiogenesis. First, angiogenic factors bind to endothelial cells, activating them (steps 1-4). This activation causes endothelial cell proliferation, migration and remodeling of the ECM (steps 5-7). These processes eventually lead to tube formation. Finally the vasculature is stabilized by recruitment of mural cells, new ECM formation, and other cell signaling (steps 7-9). (Angiogenesis Foundation 2011).

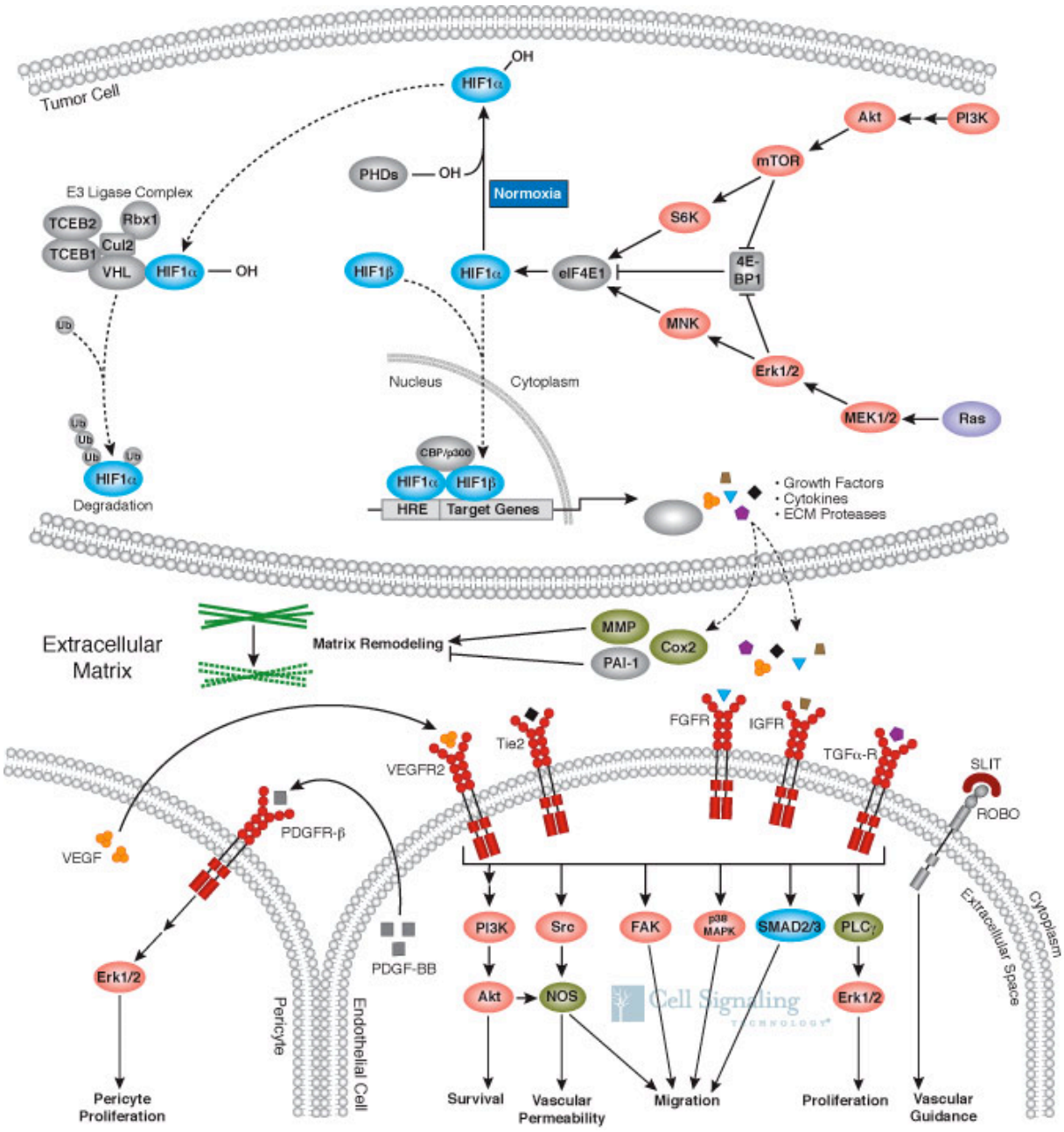

Figure 2: The cell signaling of angiogenesis. Cell signaling events induced by pro-angiogenic signals mediate angiogenesis. Growth factors, such as VEGF and FGF, can arise from cell responses to hypoxia or other physiological stimuli. These angiogenic factors bind to receptors on the endothelial cell, activating them, leading to cell responses such as proliferation, migration, vascular permeability, and increased cell survival. Angiogenic factors also stimulate the expression of MMPs, which remodel the surrounding ECM. (Cell Signaling Technology 2011)

In addition to growth factors, ECM proteins provide important pro-angiogenic and anti-angiogenic signals. Protease activity by MMPs releases angiogenic factors sequestered in the ECM (Rifkin et al. 1999, Burbridge et al. 2002). Synthesis of some ECM proteins (such as fibronectin) promotes angiogenesis (Mao and Schwarzbauer 2005), while other proteins (such as laminins) promote maturation of vessels, keeping endothelial cells in a quiescent state (Sorokin et al. 1997, Eble and Niland 2009).

2.1.4 The effect of hypoxia on angiogenesis in vivo

Hypoxia, or very low oxygen tension (often $\leq 1\%O_2$), is a particularly potent stimulator of angiogenesis. In proliferative tissues, such as wounds or tumors, rapidly dividing cells become increasingly distanced from capillaries, and eventually reaching the limit of oxygen diffusion. When oxygen levels drop markedly, cells experience respond via hypoxia inducible factors (HIFs), which are upregulated in low oxygen in almost all mammalian cells (Chandel and Budinger 2007).

The pathway that regulates HIF-1α is well understood (Figure 3). The HIF complex is a dimer consisting of HIF-1α (or HIF-2α) and HIF-1β subunits, which are constitutively expressed in the cell. In normoxia, however, HIF-1α is rapidly degraded. In the presence of oxygen, prolyl hydroxylase domain proteins (PHDs) hydroxylate proline residues, which target HIF-1α for ubiquitination. The ubiquitinated HIF-1α is subsequently degraded by a proteasome. Additionally, oxygen signals activate factor inhibiting HIF-1 (FIH-1), which inhibits P300/CPB binding and consequently expression of hypoxia responsive elements (HREs). In low oxygen conditions, PHDs and FIH-1 are inactivated, causing upregulation of HREs (Kaluz et al. 2008, Chandel and Budinger 2007). Therefore, HIF-1α is regulated at a post-translational level.

These HIFs induce activity of HREs. Although many of the downstream effects of HREs are poorly understood, it is well established that the HIFs are transcription factors of many angiogenic proteins, such as VEGF (Carmeliet 2003).

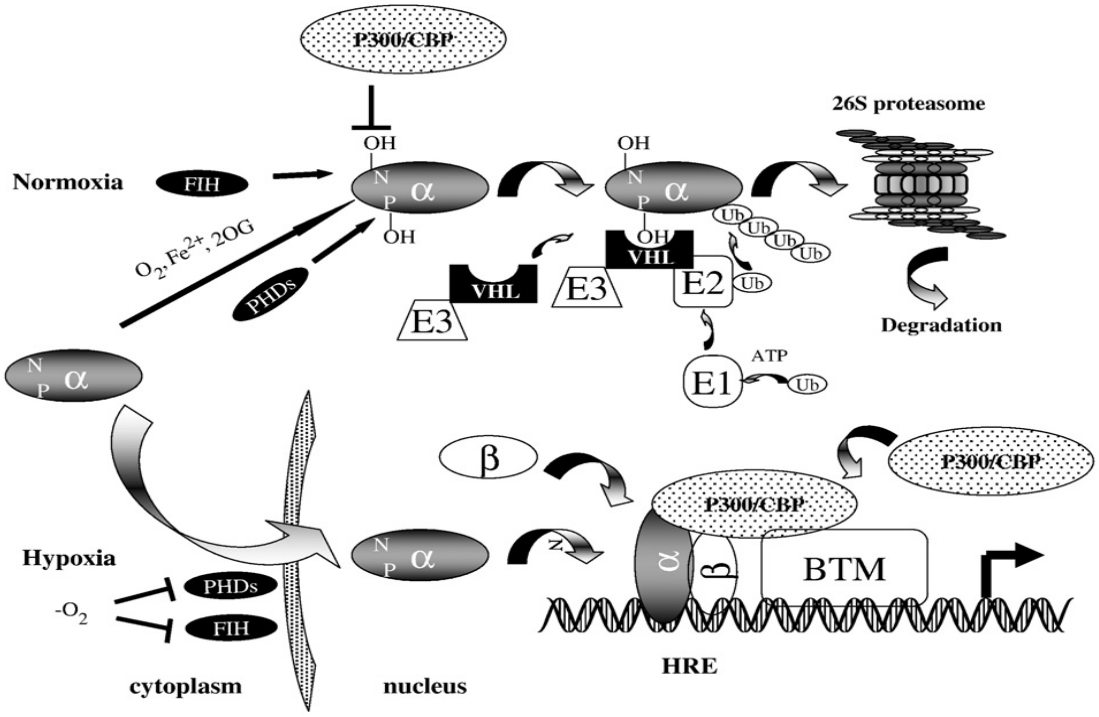

Figure 3: Regulation of HIF-1α. HIF-1α is regulated by oxygen concentrations at a post-translational level. (Kaluz et al. 2008).

2.1.5 Vasculogenesis in vivo

Whereas angiogenesis occurs via terminally differentiated endothelial cells, vasculogenesis is mediated by differentiation of endothelial progenitor cells (EPCs) into endothelial cells (Figure 4). Vasculogenesis is known to occur both in the embryo and in adults, and it is induced via similar signaling molecules to angiogenesis (such as VEGF and FGF) (Ferrara 1996, Carmeliet 2003). At early stages of embryonic development, vasculogenesis occurs through the merging of blood islands. Blood islands are made up of hemagioblasts, which differentiate into the precursors of blood cells (hematopoietic stem cells, HSCs) and precursors of blood vessels (EPCs). As this differentiation happens, blood islands merge, and the outer layer, which differentiates into angioblasts and eventually to EPCs, forms a lumen. The inner layer of the blood island differentiates into HSCs (Furguson et al. 2005). After EPCs differentiate into endothelial cells, further neovascularization may undergo via angiogenesis (Demir et al. 2007).

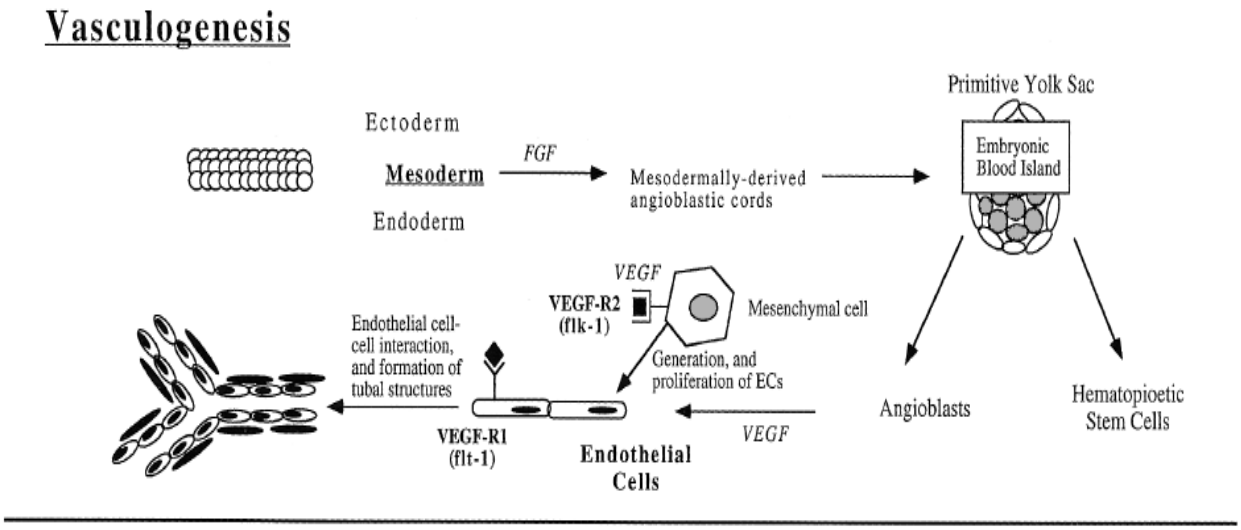

Figure 4: Vasculogenesis in embryonic development. In the embryo, vasculogenesis occurs by the fusion of blood islands and differentiation of hemangioblasts into angioblasts and HSCs. Angioblasts eventually differentiate into endothelial cells, forming vascular structures *de novo*. VEGF and FGF, which are essential for angiogenesis, also play a major role in mediating vasculogenesis. (Cines et al. 1998)

The mechanism by which vasculogenesis in adults is distinct from embryonic vasculogenesis. In adults, blood marrow-derived EPCs circulating in the blood are recruited to sites of neovascularization and form vessels through *in situ* differentiation. However, angiogenesis is the main mechanism by which neovascularization occurs in adults Adams and Alitalo 2008). While our system attempts to create neovascularization by *de novo* creation of blood vessels as occurs in vasculogenesis, the mechanism is likely distinct from vasculogenesis because of our use of adult endothelial cells.

2.2 Current approaches in engineering vascularized tissues

Due to the importance of vascularization for the survival of most tissues, there has been an enormous effort towards growing vasculature in tissue engineered constructs. To date, the two main approaches have been (1) the promotion of the host vasculatures invasion of implanted tissues and (2) the development of *de novo* vascular systems in engineered tissue, referred to as pre-vascularization (Rivron et al. 2008).

2.2.1 Angiogenic invasion by host vasculature

A technique to improve vascularization in engineered tissue transplants involves the promotion of angiogenic invasion by the host on the implanted tissue. One well documented example of this technique is the delivery of growth factors from the scaffold of the implanted construct, directing angiogenic invasion into the new tissue (Lovett et al. 2009).

2.2.2 Pre-vascularization

Prevascularization is the technique of creating *de novo* vascular networks for *in vitro* engineered tissue prior to implantation. This technique is achieved via cultures of endothelial cells or stem cells, often with co-cultures including other tissue related cells (eg. a co-culture

of endothelial cells and adipocytes to create an engineered adipose tissue) (Rivron et al. 2008). The goal is to create a tissue with a vascular network sufficient for survival and development after *in vivo* implantation. In the past decade, significant advances have been made in the development of pre-vacularized tissues.

One early example of a pre-vascularized tissue was an engineered skin tissue, created by Black et al. in 1998. In their study, a tri-culture of keratinocytes, fibroblasts and human umbilical vein endothelial cells (HUVECs) were grown on collagen scaffolds. In the tissue culture, the endothelial cells organized into a tubular network (Black et al. 1998). A later study by the same group successfully implanted an engineered skin tissue in mice. Histological results showed that the capillary-like network from the engineered skin successfully inosculated with the host's capillary network under the dermis after 4 days (Tremblay et al. 2005).

2.2.3 Prevascularization of engineered adipose tissue

A recent study in our lab illustrated successful vascularization of adipose tissue. A co-culture of HUVECs and human adipose-derived stem cells (hASCs) was grown on a 3D porous silk scaffold. A monoculture of HUVECs was cultured on the scaffold for one week prior to seeding of hASCs on the scaffold. The HUVEC and hASC co-culture was grown on the scaffold for two weeks. Confocal microscopy showed progressive organization of both cell types with time, and histology confirmed lumen formation by HUVECs at day 14 of the co-culture (Kang et al. 2009).

In this study we will focus on the optimization of the endothelial cell monoculture prior to addition of a second cell type. We attempt to improve endothelial cell proliferation, ECM formation and cell organization of the monoculture by optimization of oxygen tension.

2.3 *In vitro* tools for vascular development

The choice of scaffolding materials and cell types are important considerations for any tissue engineering project. For tissue engineering purposes, scaffolds must have appropriate structural, physical, and degradation properties for the tissue of interest (Langer and Vacanti 1993). For example, adipose tissue and vascularized stents may require vastly different scaffold properties. Additionally, the materials used in the scaffold must be biocompatible.

Moreover, many chemical and physical factors can be used to control the physiology of endothelial cells. As mentioned in section 2.1.3, chemical stimuli such as growth factors mediate endothelial cell proliferation and organization into vascular structures. Oxygen environment is also a very important regulator of endothelial cell proliferation via growth factors as well as ECM synthesis and organization. Physical stimuli such as shear stress, are essential to proper endothelial cell physiology. Tissue engineers have explored the use of many such stimuli for *in vitro* cell and tissue culture.

2.3.1 Endothelial cells: HUVECs and HMVECs

HUVECs and HMVECs are the two most extensively used endothelial cells for *in vitro* angiogenesis models, mostly due to their ease in isolation and *in vitro* culture. Both HUVECs and HMVECs are terminally differentiated cells with ample proliferative potential (Bagley et al. 2003) While both cell types are well established in the field of *in vitro* angiogenesis, HMVECs have shown better proliferative potential, as they remain viable at higher passage numbers (Augustin 2003). Both HUVECs and HMVECs were used in this study.

2.3.2 Porous silk scaffolds and silk tube scaffolds

The use of silk fibroin scaffolds in tissue engineering has been of particular interest due to the biocompatibility, structural properties and control of degradation rate of engineered silk. Silk has been used for biomedical sutures for centuries, illustrating its potential use as a biomaterial (Altman et al. 2002). Silk scaffolds showed low immune responses when implanted in rats, and degradation time of scaffolds ranged from 2-6 months (aqueous silk scaffolds) to up to a year (HFIP solvent based scaffolds) *in vivo* (Wang et al. 2008). Additionally, the use of gel spinning of silk tubes has produced tubes with useful properties. Some properties include control of pore size and weaving pattern, different mechanical properties, and control of cell attachment via synthetically attached cell binding motifs (Lovett et al. 2008).

Previous studies in our laboratory on vascular grafts and vascularized adipose tissue have shown the feasibility of seeding silk scaffolds with endothelial cells. Our lab has also successfully seeded human umbilical vein endothelial cells (HUVECs) on porous 3D scaffolds in a study on an *in vitro* 3D model of vascularized adipose tissue coculture (see 2.2.3) (Kang et al. 2009). Aqueous-based 3D porous silk scaffolds and porous silk tube scaffolds will be used in this study. Additionally, we created silk tube-scaffold "construct," which was a porous silk scaffold with a tube scaffold through the center. If this construct were to be successfully vascularized, the tube could be sutured into a large blood vessel, bringing blood flow immediately to a transplanted tissue.

2.3.3 Hypoxia

In vitro studies have shown that hypoxia ($\leq$ 1% O_2) is a potent activator of neovascularization in both angiogenesis and vasculogenesis pathways. One study with

HUVECs showed that HIF-1α was upregulated in 1% conditions compared to 21% oxygen conditions. Tube formation was found in cultures 1% oxygen conditions in media without growth factors, while no tube formation in 21% oxygen in the same media; in media with growth factors, however, tube-like structures formed under both oxygen conditions, which suggests that HIF-1α may upregulate growth factors. Moreover, bFGF-neutralizing antibodies completely inhibited angiogenesis in hypoxic samples, which suggests that basic fibroblast growth factor (bFGF) expression was induced via the HIF-1α response (Calvani et al. 2006).

However, while short term exposure to hypoxia stimulates angiogenesis *in vitro* (in the absence of growth factors), chronic exposure to hypoxic environments hinders angiogenesis. One study in HUVECs showed that while acute hypoxia (1% O_2 culture for 24 hrs, 21% oxygen for 13 days) mildly stimulated tube formation compared to 21% oxygen controls, chronic hypoxia (1% O_2 for 14 days) severely inhibited tube formation (Zhang et al. 2009). Additionally, chronic hypoxia significantly retarded HUVEC proliferation compared to acute hypoxia and normoxia cultures (Zhang et al. 2009). An *in vitro* study in HMVECs indicated that cells grown in hypoxic conditions (<1% O_2) exhibited significantly higher apoptotic activity, as caspase 3 and caspase 7 were upregulated compared to cells cultured in 5% oxygen and 21% oxygen (Decaris et al. 2009). These data suggest that the use of hypoxia for tissue engineering with endothelial cells in the presence of growth factors may not be very beneficial, especially for long term cultures. These data also indicate that inadequate oxygen diffusion to the center of an endothelial tissue culture may be a potential problem due to prolonged hypoxia.

2.3.4 Low oxygen (~5% O_2)

It has been estimated that normal *in vivo* oxygen concentrations of tissues reflect extracellular oxygen tensions of 4%-6% (Maisch et al. 1998, Faller 1999, Zhou et. al. 2000). As is pointed out by Zhou et al. 2000, although low oxygen tension (5% oxygen) could be considered *hypoxic* to 21% oxygen, the fact that a 5% oxygen environment closely resembles *in vivo* oxygen concentrations, suggests that 21% oxygen is *hyperoxic* to a *normoxic* 5% oxygen environment. Zhou et al. thus hypothesized that 21% oxygen may negatively affect endothelial cell culture as compared to 5% oxygen (Zhou et al. 2000). Interestingly, several recent studies in *in vitro* culture of endothelial cells under low oxygen conditions (4%-5% oxygen) has been shown to increase proliferation, stimulate ECM development, and improve lumen and tube formation as compared to 21% oxygen controls (Zhao et al. 2008, Decaris et al. 2009, Zhou et al. 2000).

Several studies have demonstrated that low oxygen tension increased proliferation of endothelial cells in 2D cultures, as compared to 21% oxygen and hypoxic ($\leq$ 1% oxygen) controls. HUVECs grown in cell culture with growth factors showed statistically higher cell number in 5% oxygen after 2 and 3 days of culture, as compared to 20% oxygen counterparts (Zhao et al. 2008). Moreover, after three days of culture, significantly higher number of cells exhibited BrdU incorporation in 5% oxygen as compared to 20% oxygen, suggesting that low oxygen tension (5% O_2) facilitates proliferation of endothelial cells (Zhao et al. 2008). HMVECs in cell culture with growth factors had a cell doubling rate of 2.4 $\pm$ 0.07 days under 4% oxygen compared to the 3.58 $\pm$ 0.3 day doubling rate of HMVECs grown under 21% (Decaris et al. 2009). Additionally, while HMVECs grown in 1% oxygen showed significantly higher caspase 3 and caspase 7 activity compared to 21% oxygen controls, there

was no difference in caspase activity between cells grown 5% oxygen and 21% oxygen, which suggests that low oxygen tension (5% oxygen) does not elicit an apoptotic response (Decaris et al. 2009).

In addition to stimulating proliferation in growth media, low oxygen tensions have been showed to increase ECM development in endothelial cell monocultures. One study in microvascular endothelial cells showed that cell culture in 5% oxygen showed higher mRNA levels of platelet endothelial cell adhesion molecule -1 (PECAM-1 or CD31) and collagen IV as compared to 21% oxygen controls (Zhou et al. 2000). Another study of HUVEC monocultures showed that cells grown in 5% oxygen created elaborate fibrin and collagen IV networks, while cells grown in 20% oxygen created a far less organized network with limited amounts of fibronectin and collagen proteins. Western blot analysis showed significantly higher fibronectin and laminin in the 5% oxygen cultures as compared to the 20% oxygen cultures (Zhao et al. 2008).

Moreover, low oxygen has been showed to improve endothelial organization and lumen formation in 2D endothelial cell cultures, as compared to 21% oxygen controls. In one study, HMVECs grown in the presence of growth factors in 1% or 5% oxygen formed closed, well rounded tubes made up of multiple cells, while the cells grown in 21% oxygen formed polygonal tubes, many of which were not closed (Decaris et al. 2009). A second study showed similar results in HMVECs showed that cells grown in a collagen matrix under 5% oxygen created lumen-like structures more quickly than cells grown under 21% oxygen; however, after a certain time point, there were no significant differences in tube-like formation (Zhou et al. 2000).

These studies suggest that culturing cells in low oxygen environments (~5% oxygen) could improve the proliferation rates, the ECM networks, and lumen formation of endothelial cells *in vitro*. However, nearly all of the experiments were performed in 2D cultures. We explored the potential of using a 5% oxygen system for the monoculture of HUVECs or HMVECs in a 3 dimensional porous silk scaffold, with cells grown in 21% oxygen as a control. We assessed the potential of low oxygen pre-culture using several methods: DNA quantification assays, which were used to monitor cell number; histology and confocal microscopy, which were used to assess cell organization; and real time RT-PCR, which assessed the cells' synthesis of ECM adhesion proteins in response to low oxygen.

2.3.5 Dynamic cultures

Dynamic cultures induce mechanical forces on cells and stimulate the transfer of nutrients, gasses and growth factors. Forces, such as shear stress, are an important regulator of endothelial cell function. *In vivo*, endothelial cells are exposed to shear stresses ranging from roughly 10-20 dynes/cm^2 in arteries (Nerem et al. 1998) to 1-5 dynes/cm^2 in capillaries (Cucullo et al. 2005). Laminar shear stress affects endothelial cell morphology and orientation, causing cells to organize in the direction of flow due to directional rearrangements of the actin cytoskeleton (Wong et al. 1983, Ando and Yamamoto 2011).

Dynamic cultures have been used in many *in vitro* systems. Cultures that induce significant shear stresses (5 dynes/cm^2 – 20 dynes/cm^2) have been used as a pre-conditioning for endothelialized vascular grafts, as it improves cell retention in these grafts when implanted *in vivo*. While this shear pre-conditioning can cause significant decellularization in the *in vitro* culture, gradual increases in shear stress have improved cell retention (Inoguchi

2007, Ott and Ballerman 1995). A recent study by Frerich et al. has shown the feasibility of pulsatile perfusion for pre-vascularization of 3D cultures. Co-cultures of HUVECs and adipose tissue stromal cells in gelatin tube scaffolds had improved capillary networks (stemming from the interior lumen of the tube) when they were exposed to pulsatile flow, as compared to static conditions (Frerich et al, 2008).

Moreover, dynamic systems are important for the delivery of oxygen, nutrients, and cell signals to endothelial cells. Studies have suggested that flow is important endothelial cell survival, as cease of flow has been linked to capillary regression and apoptosis due to decrease in VEGF migration (Meeson et al. 1996, Meeson et al. 1999). A recent *in vitro* study in HMVECs showed that low levels of flow are essential for capillary angiogenesis, as it improves transfer of VEGF released from the ECM (Helm et al. 2005). Moreover, inadequate transport of nutrients and (especially) oxygen has been a major challenge in tissue engineered vascular constructs. The use of rotation (Dutt et al. 2003) and (more successfully) perfusion bioreactors have been used to overcome these mass transfer challenges (Grayson et al. 2008, Lovett et al. 2009).

2.3.6 Low oxygen tension may improve cellular retention under shear stress

Interestingly, one recent study suggested that endothelial cells pre-cultured in low oxygen exhibited a higher cellular retention rate when exposed to shear stress. Zhao et al. 2008 pre-cultured endothelial cells in 5% oxygen for 3 days on PET films, resulting in significantly higher cell retention when exposed to high shear stress (ranging from 5-20 dynes/cm^2) as compared to 20% oxygen controls. They postulated that the increased cell retention of the low oxygen pre-treated endothelial cells was due to increased secretion of

ECM proteins (such as collagen IV and fibronectin), increased synthesis of cell adhesion proteins (such as PECAM-1), and more extensive ECM networks, which were observed via immunofluorescence. This study highlighted the potential use of low oxygen treatment for increasing endothelialization in cultures exposed to shear stress (Zhao et al. 2008).

In our study, we assess the potential use of low oxygen (5% oxygen) treatment as a tool to increase endothelialization of HMVEC monocultures in 3D silk scaffolds under dynamic culture. Although our simple rotation culture system may not be useful for pre-endothelialization because of the non-linear shear forces on the cells and because of unequal exposure of shear stress throughout the scaffold, it assesses the use of low oxygen as a means to increase cell retention on silk scaffolds. Low oxygen environments to improve endothelialization could be applied to more complex perfusion systems.

Chapter 3: Hypothesis and Specific Aims

3.1 Hypothesis

The central hypothesis of this study is that endothelial cell proliferation and organization in static 3D tissue cultures, as well as endothelial cell retention on 3D scaffolds in dynamic tissue cultures, are improved at low oxygen (~~% O_2) as compared to 21% O_2 cultures. We also hypothesize that, under short term (1-2 week) low oxygen (5% O_2) tissue cultures, the porous silk scaffold will allow sufficient diffusion of oxygen to cells in the center of the scaffold such that these cells will not experience hypoxia (< 1%O_2), and therefore will not contain high levels of HIF-1α compared to 5% oxygen 2D controls. We hypothesize that increased levels of ECM adhesion proteins, such as PECAM-1/CD31, will be present in 5% oxygen tissue cultures, compared to 21% oxygen controls.

3.2 Specific aims

Aim 1: To improve endothelial cell proliferation and organization in 3D porous silk scaffolds by manipulating oxygen tension. Endothelial cell proliferation will be assessed using DNA quantification, which provides a quantitative measure of cell number in the tissue cultures. Cell organization will be analyzed using CD31 immunohistochemistry for these tissue cultures.

Aim 2: To assess the effect of oxygen tension on endothelial cell adhesion to silk scaffolds in dynamic cultures. The cellular retention will be assessed via DNA quantification assays of tissue cultures in dynamic conditions.

Aim 3: To determine if low oxygen tissue culture caused a hypoxia response in some cells in the 3D scaffold. This will be assessed by a HIF-1α western blot on the tissue cultures.

Aim 4: To determine if PECAM-1/CD31 levels in endothelial cells in tissue cultures were affected by oxygen tension. This will be examined using quantitative RT-PCR of CD31 in the tissue cultures.

Chapter 4: Materials and methods

Materials

All tissue culture materials and PicoGreen dsDNA assay was purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. HUVECs, HMVECs, Endothelial Basal Media (EBM-2), Endothelial Growth Medial Bullet Kits (EGM-2MV and EGM-2 bullet kits) were purchased from Lonza Inc. (Walkersville, MD). *Bombyx mori* silkworm cocoons were supplied by Tajima Shoji Co. LTD, (Yokohama, Japan). All chemical reagents used in silk processing were provided by Sigma-Aldrich (St. Louis, MO).

4.1 Preparation of aqueous silk fibroin solutions for scaffolds

Silk solution was prepared as described in Lovett et al, 2007. Briefly, silk fibroin solutions of 6% (wt/vol) were made using *Bombyx mori* silkworm cocoons. The fibroin was extracted from cocoons in a boiling 0.02 M sodium carbonate solution for 30 minutes, rinsed in deionized water, dried overnight, dissolved in 9.3 M lithium bromide, and dialyzed against deionized water using a Slide-a-lyzer dialysis cassette (molecular weight cutoff MWCO, 3500 Daltons, Pierce, Rockford, IL) for 48 hours. The resulting solution of 6-8% wt/vol fibroin was further diluted to 6% (wt/vol). The silk solution was stored at 4°C.

4.2 Preparation of 3D silk scaffolds

Silk scaffolds were prepared by adding 51g of sieved granular NaCl (granulated, 500 - 600μm) to 28.5 mL silk fibroin solution in a 8.5 cm diameter Petri dish coated in Teflon tape. The dishes were covered and kept in a fume hood at room temperature for 72 hours. Open containers were rinsed against distilled water for 48 hours to leach out the NaCl. The scaffolds were removed from the dishes and an 8mm biopsy punch was used to obtain

scaffolds. The final dimensions of the scaffold were 8 mm in diameter by 4 mm in height. The scaffolds were first dried, then autoclaving prior to cell seeding.

4.3 Preparation of concentrated silk solution for tubes

Concentrated silk solution was prepared as described in Lovett et al. 2007. Briefly, silk fibroin solutions of 20% (wt/vol) was made by dialyzing 6-8% silk fibroin solution (in a Slide-a-lyzer dialysis cassette) against 10% (wt) polyethylene glycol (PEG) solution for 24 hours, yielding a 20 – 30% (wt/vol) solution. The silk concentration was determined, and an appropriate volume of water was added to normalize the silk concentration to 20% (wt/vol). The resulting solution was blended with 6% (wt) polyethylene oxide (PEO) solution to form 80/20 (wt%) silk/PEO solution.

4.4 Preparation of porous silk tubes

Porous silk tubes were prepared as described in Lovett et al 2007. Briefly, silk tubes were prepared by dipping stainless steel mandrels (1.6 mm diameter) in 80/20 (wt%) silk fibroin/PEO solution until evenly coated and dipped in methanol to induce antiparallel β sheet formation in the coated silk. This dipping procedure was repeated 3 times for each silk coated mandrel, and the silk was dried overnight. The silk tubes were removed from the mandrel using a dilute detergent-water solution; the tubes were rinsed thoroughly to remove any residual detergent left on the tube. The tubes were cut to a length of 1 cm. The tubes were dried and autoclaved prior to cell seeding.

4.5 Tube-scaffold construct preparation

The construct was assembled under sterile conditions. Autoclaved tubes and scaffolds were soaked in EGM-2 medium for 1 hour. A tube was placed through an 18 gauge needle; the tip of the tube-coated needle was pierced through the center of a 8x4 mm (dxh) 3D

scaffold. Forceps were used to gently pull the pierced scaffold to the center of the tube. The resulting tube-scaffold was removed from the needle and placed in EGM-2 media for one hour prior to seeding.

4.6 Cell culture

Human microvascular vein endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs) were cultured according to manufacturer's protocol. HMVECs were grown in optimized growth media (EGM-2), consisting of Endothelial Basal Medium-2 supplemented with EGM-2-MV Bullet kit. HUVECs were grown in optimized growth media (EGM-2), which is composed of Endothelial Basal Medium-2 supplemented with EGM-2 bullet kit. The cells under normoxic conditions were cultured at 37°C, 5% CO_2/95% air (~20% O_2), and 95% relative humidity. The cells under a low oxygen tension were cultured with the same protocol as in 2.5, but at 5% CO_2/ 5% O_2, 90% N_2 conditions. The media was changed 2 times a week. The cells were passaged at approximately 90% confluence using Trypsin-EDTA (0.25% trypsin with 1 mM EDTA).

4.7 Cell seeding

The media-soaked scaffolds were placed in dry 12-well plates. The scaffolds were seeded with 20 μL doses of cell suspensions repeated three times. Cells were allowed to attach for approximately 3 hours; each scaffold was seeded with 0.75 x 10^6 cells. Two mL of media was added to each scaffold (one scaffold/well of 12 well plate) and was replenished 2 times a week.

4.8 Experimental tissue culture—static culture

The normal oxygen tension experimental groups were cultured at 37°C, 5% CO_2/95% air (~20% O_2), and 95% relative humidity. The low oxygen tension experimental groups

were cultured at 37°C, 5% CO_2/5% O_2/ 90% N_2, and 95% relative humidity. Media was replenished 2 times a week.

4.9 Experimental culture—rotation culture

After seeding, cells were allowed to attach for 24 hours and allowed to proliferate for 5 days. The seeded scaffolds were placed on a Lab Rotator Analog Type 2100A (LW Scientific, Lawrenceville, CA) orbital shaker in the appropriate incubator. The rotation frequency was increased linearly for 4 days, ultimately reaching a maximum shear force of 4 dynes/cm^2. The rotation speed was increased in a gradual, stepwise manner, increasing the shear force by 1 dynes/day for 4 days. Otherwise, the cells were cultured as described in section 3.8.

4.10 Experimental culture—rotation culture: calculation of rotation frequency

The rotation frequency was calculated using the following equation:

$$\tau_{\max} = \alpha\sqrt{\rho\eta}(2\pi f)^3$$

where α is orbital radius (1.0 cm), ρ is density of the media (1.0 g/cm^3), η is viscosity of the media (0.01 poise), and f is rotation frequency (McIlhenny et al. 2010). A rotation frequency of about 111 rotations per minute corresponded to a maximum shear force of 4 dynes/cm^2.

4.11 Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR on tissue culture samples was done as described in Choi et al., 2010. Briefly, total RNA was extracted from tissue cultures using Trizol reagent (Invitrogen, Carlsbad, CA). Scaffolds were placed in Trizol and frozen at -80°C. After thawing, scaffolds were chopped to release cells from the center of the scaffolds, and the RNA extracts were centrifuged at 13,000 RPM for 10min at 4°C. The supernatants were obtained, and RNA was isolated with the Quiagen RNeasy kit according to supplier's instructions (Quiagen, Hilden,

Germany). Reverse transcription was performed using high-capacity cDNA reverse transcription kit following manufacturers protocols. Primers and probes from TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) were used for target genes HIF-1α, VCAM-1, and CD31, and Ct values were normalized to the housekeeping gene GAPDH. Real-time RT-PCR was performed using a Stratagene Mx 3000P Real-Time PCR System (Strategene, Santa Clara, CA). Reactions were performed at 50°C for 2min, 95°C for 10 min, and then 50 cycles of 95°C for 15 seconds (to denature the DNA) and 60°C for 1 minute (for DNA synthesis) were performed. (Choi et al., 2010).

4.12 PicoGreen dsDNA quantification assay

The DNA content (PicoGreen) assay was done by manufacturer's protocol. Briefly, samples were analyzed using the Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA). Samples were removed from media and placed in a clean 12 well plate. Each sample was washed twice with 3mL PBS and 1mL of CyQuant cell lysis buffer was added to release the DNA into solution. The samples in the cell lysis buffer were stored at -80°C prior to the assay. The samples were thawed and the scaffolds were finely chopped to release the cells from the center of the scaffolds. Samples were collected and centrifuged (12,000 rpm, 10 min, 4°C), and the supernatant was collected; the supernatants were analyzed in triplicates. The DNA content was determined fluorometrically at 480nm excitation/ 520nm emission using a Molecular Devices Spectral Max Gemini EM Spectrofluorometer (Molecular Devices, Sunnyvale, CA). The DNA content of each sample was determined by the interpolation of a standard curve from lambda DNA in 10mM Tris-HCl (pH 7.4), 5 mM NaCl, and 0.1 mM EDTA (Kang et al. 2009).

4.13 CD31 Immunohistochemistry Staining

CD31 immunohistochemistry and H&E staining were prepared as described in Kang et al., 2009. Briefly, scaffolds were fixed in 10% formalin and stored at 4°C before paraffin processing and embedding. Fixed samples were dehydrated with a series of three ethanol washes (80%, 95%, and 100%) and three xylene washes. Samples were treated for paraffin for 1.5 h in vacuum. After this treatment, the samples were embedded in paraffin, and allowed to cool overnight. The paraffin-embedded samples were then sectioned (10 µm sections) and an anti-CD31 staining procedure was performed (Invitrogen, Carlsbad, CA). The sections were washed with xylene three times, dehydrated with three ethanol washes (100%, 95%, and 70%), and underwent heat treatment in 0.01 M citric acid buffer (pH 6). Next, endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol for 15 min, unspecific binding sites were blocked with goat serum (1:20 in 1.5 % milk), and samples were incubated in a primary anti-human CD31 antibody overnight. A biotin-labeled goat anti-mouse secondary antibody was allowed to attach to sections for 1 hour. To develop the reaction, the samples were treated with 1:150 strepavadin-peroxidase complex in 1 % BSA for 30 min. Then the samples were incubated in a DAB/urea solution for 10 minutes. Finally the samples underwent hematoxylin counterstaining and were mounted (Kang et al. 2009).

4.14 Staining of HMVECs with Vybrant DiD Stain

HMVECs were stained according to company protocols. Vybrant DiD stain was added to a cell suspension with 1 x 10^6 cells/ml PBS, creating a final concentration of 5µl of DiD dye per 1 mL of suspension. The suspension was gently mixed and allowed to incubate at 37°C for 20 minutes. The suspension was pelleted by centrifugation at 1500 rpm for 5

minutes at 37°C, the supernatant was discarded, and the cells were resuspended in EGM-2MV media. This wash step was repeated twice and the final cell suspension was used for seeding.

4.15 Staining by Vybrant DyeCycle Ruby Dye

Vybrant DyeCycle Ruby Dye (Invitrogen, Carlsbad, CA) was added to the tissue culture sample, which contained 2mL of EGM-2MV media, creating a 5µM solution of the dye in the media. The stained tissue culture was incubated at 37°C and imaged without washing.

4.16 Confocal microscopy

Confocal microscopy was used to analyze cell organization in the scaffolds. Confocal imaging was performed using a Leica DMI RE2 confocal microscope (Leica, Wetzlar, Germany). Cells dyed with vibrant DiD stain were imaged with 644/670 (ex/em) and Vybrant Ruby DyeCycle stained cells were imaged with 488/670 (ex/em).

4.17 Western Blotting

Western blot was performed as described in Subramanian et al. 2010. Briefly, cells were lysed using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) and supplemented with Halt Protease Inhibitor (Pierce Biotechnology, Rockford, IL) at 4°C for 30 minutes. The cell lysates were centrifuged at 14,000 rpm for 15 minutes, and the supernatant was collected to obtain the soluble protein extract. The total protein content was quantified using the Bradford Assay (BioRad, Hercules, CA). Protein samples were heated to 70°C for 10 minutes in Nupage LDS buffer. Equal total protein samples were loaded into a 4-12% bis-tris gel (Invitrogen, Carlsbad, CA) and electrophoresed. Proteins from the gel were transferred to a polyvinylidenefluoride membrane (Invintrogen, Carlsbad, CA). The

membrane was blocked from non-specific binding using a 5% milk solution for HIF-1α. The membrane was then probed for HIF-1α using ab1 (1:1000) (Abcam, Cambridge, MA) and using goat anti-mouse horseradish peroxidase conjugate (1:3000) (Santa Cruz Biotechnology, Santa Cruz CA). For GAPDH, the membrane was blocked using and 5% BSA, and then probed for GAPDH using ab9484 (1:2000) (Abcam, Cambridge, MA) and goat anti-mouse horseradish peroxidase conjugate (1:5000) (Santa Cruz Biotechnology, Santa Cruz CA). (Subramanian et al. 2010).

4.18 Research Design: Construct, tube and scaffold tissue cultures under various oxygen tensions

HUVECs were cultured under 5% or 21% oxygen until confluence was reached. 0.75 x 10^6 cells were seeded on a silk tube, silk scaffold or a tube-scaffold construct and maintained at 5% or 21% oxygen for 2 weeks (Figure 5). There were three oxygen groups: HUVECs maintained in 5% oxygen, 21% oxygen, and cells tissue cultured in 5% oxygen but seeded with HUVECs that were expanded in 21% oxygen (the "21% to 5% oxygen" group). Cells were sacrificed for DNA quantification assay and CD31 immunohistochemistry after the two week culture. N=3 for each experimental group.

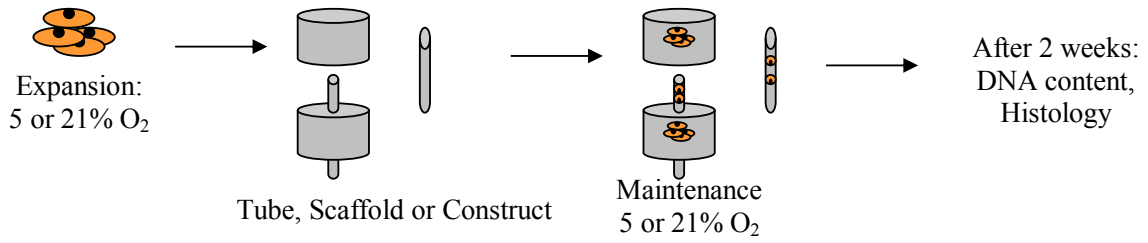

Figure 5: Schematic diagram of the experimental design for *Construct, tube and scaffold tissue cultures under various oxygen tensions*

4.19 Research Design: Tissue cultures under dynamic or under static conditions in various oxygen conditions

HMVECs were cultured in 5% or 21% oxygen until confluence, and 0.5 x 10^6 cells were seeded onto a silk scaffold, and maintained under 5% oxygen or 21% oxygen for two weeks (Figure 6). One group was cultured under static conditions, while the other was cultured under dynamic conditions. The dynamic culture was seeded at day 0 and allowed to grow in static culture until day 6, when surface shear stress (induced by rotation culture) was increased by 1 dyne/cm^2 for 4 days, reaching a max shear stress of 4 dynes/cm^2 from day 6 until day 14. Samples were sacrificed at day 0 as a seeding control for the DNA quantification assay, and after 2 weeks for DNA quantification, histology, and RT-PCR of HIF-1α and PECAM-1. For the DNA quantification data, DNA content was normalized to seeding controls by dividing the sample DNA content by the average DNA content of the seeding controls.

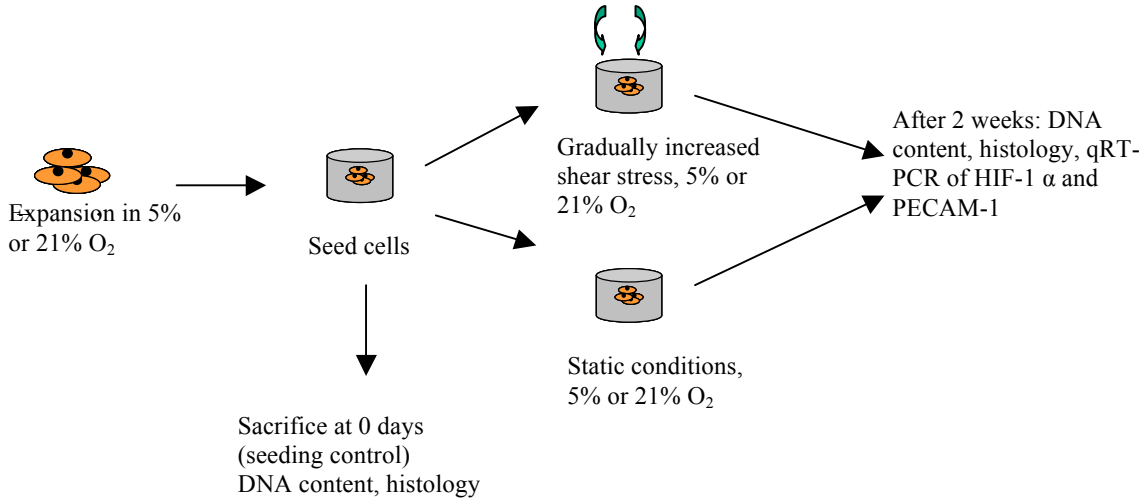

Figure 6: Schematic diagram of the experimental design for *Tissue cultures under shear stress or under static conditions in various oxygen conditions*

4.20 Research design: Tissue cultures under various oxygen tensions

HMVECs were cultured in 5% or 21% oxygen until confluence, and 0.75 x 10^6 cells were seeded onto a silk scaffold, and maintained under 5% oxygen or 21% oxygen for one week (Figure 7). There were three oxygen groups: HUVECs maintained in 5% oxygen, 21% oxygen, and cells tissue cultured in 5% oxygen but seeded with HUVECs that were expanded in 21% oxygen (the "21% to 5%" group). Samples were taken for DNA quantification at days 0, 3 and 7, and samples were taken for HIF-1α and PECAM-1 western blot at day 7. Additionally, HMVECs labeled with DID stain were seeded on scaffolds at 0.75 x 10^6 were imaged by confocal microscopy at day 3 and day 7. A second Vybrant DyeCycle Ruby Dye was included for the day 7 imaging samples due to difficulties in confocal imaging with the DiD dye.

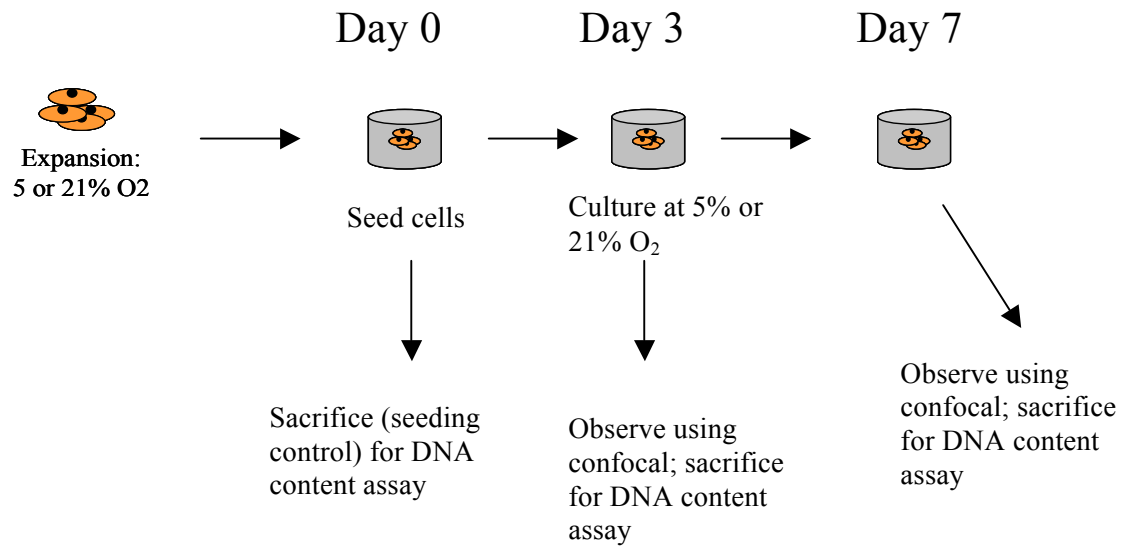

Figure 7: Schematic diagram of the experimental design for *tissue* cultures under various oxygen tensions

For the DNA quantification data, DNA content was normalized to seeding controls by dividing the sample DNA content by the average DNA content of the seeding controls. Percent increase of DNA content was calculated by dividing day 7 concentrations by the average day 3 concentrations.

4.21 Statistical analysis

All reported values were averaged and expressed as $\pm$ standard deviation. For all statistical analyses, the Levine's test for the homogeneity of variances was performed to test homoscedasticity of samples. For data with homogeneity of variance, a Student's t-test (for samples with 2 groups) or a one-way ANOVA (for samples with 3 or more groups) was performed. The Bonferroni multiple comparisons test was performed post-hoc to ANOVA. For data that did not exhibit homogeneity of variance, a Kruskal-Wallis test was performed. For all performed statistical tests, differences were considered statistically significant if $p < 0.05$. All statistical calculations were carried out using SPSS PASW Statistics Student Version 18.0 software.

Chapter 5: Results

5.1 DNA Quantification results of construct, tube and scaffold tissue cultures under various oxygen tensions

A DNA quantification assay was used to analyze cell number in various silk scaffolds (tube-scaffold construct, tube or scaffold only) under various oxygen conditions. While DNA content cannot estimate cell number, it provides a quantitative measure that can be used for relative comparison of cell number. HUVECs cultured under 21% oxygen or 5% oxygen were seeded on a silk "construct," tube or scaffold only, and tissue cultured under 21% oxygen or 5% oxygen for 14 days. A DNA quantification assay was performed on each group after 14 days (Figure 8).

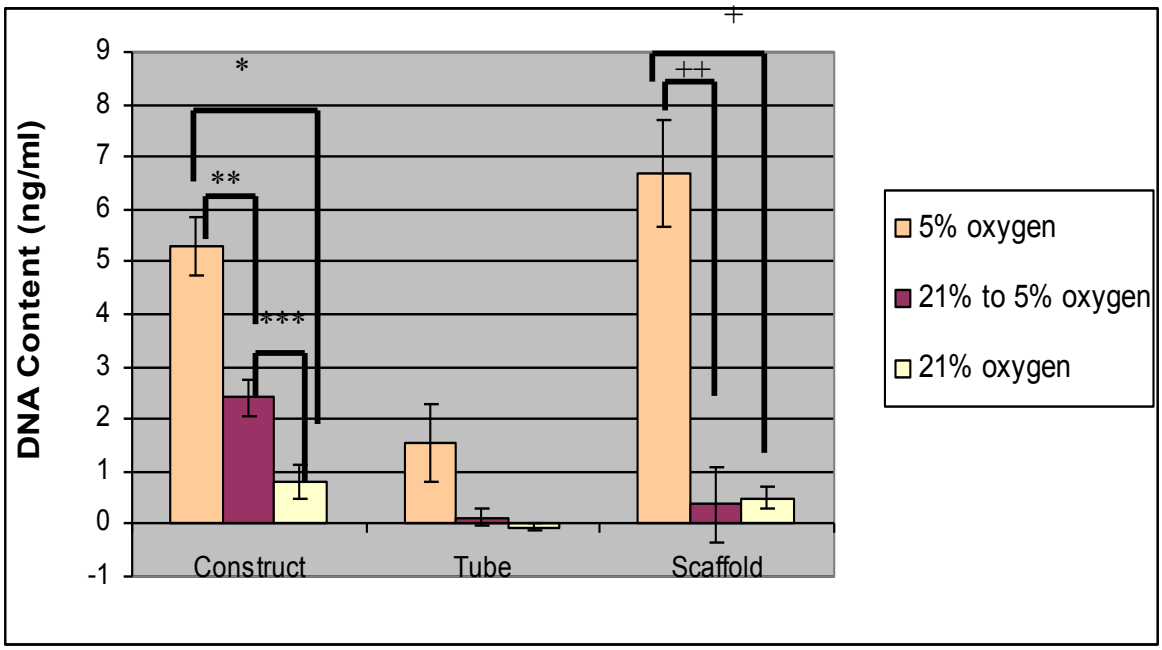

Figure 8: DNA content of 14 day tissue culture in various scaffolds and oxygen environments. Error bars represent standard deviations (N=3).

Significant differences in DNA content were found between oxygen tensions in construct tissue cultures (ANOVA, df = 2, 6, F = 87.094, $p < 0.005$) (Figure 8). The 5%

oxygen group had significantly higher DNA content compared to the "21% to 5% O_2" group (the group tissue cultured in 5% oxygen but seeded with cells cultured in 21% oxygen) (Bonferroni $p^{**} < 0.005$) and compared to the group cultured in 21% oxygen only (Bonferroni $p^{*} < 0.001$). Additionally, the "21% to 5% O_2" group had significantly higher DNA content compared to the 21% oxygen group (Bonferroni $p^{***} < 0.01$). No significant differences were observed between oxygen tensions for the silk tube tissue cultures (Independent samples Kruskal-Wallis test, $p > 0.05$).

Statistically significant differences were also found between oxygen groups cultured in the silk scaffold (ANOVA, df = 2,6, F = 63.794, $p < 0.001$) (Figure 8). Low oxygen (5% O_2) tissue cultures had significantly higher DNA content compared to the "21% to 5% oxygen" group (Bonferroni $p^{++} < 0.001$) and the 21% O_2 only tissue culture (Bonferroni $p^{+} < 0.001$). No significant differences were found between the other groups (Bonferroni $p > 0.05$).

These DNA quantification data show that the 5% oxygen tissue cultures had higher DNA content than the 21% oxygen and "21% to 5% oxygen" groups (Figure 8). These results indicate that higher cell numbers were present in the 5% oxygen tissue cultures as compared to the other cultures. However, it is important to note that the DNA quantification data was not normalized to seeding density. It is possible that seeding density was a confounding variable which caused systematic error in the data.

In addition to investigating the effect of oxygen tension on cell numbers, we were interested in the effect of the scaffold type (tube-scaffold construct, tube or scaffold only) on cell number. To assess this effect, statistical tests were performed between the scaffold groups for each oxygen tension (Figure 8).

Statistically significant differences were found between scaffold types in the 21% oxygen group (ANOVA, df = 2,6, F= 10.656, $p< 0.05$), in the "21% to 5% oxygen" group (ANOVA, df = 2,6, F=21.625, $p< 0.05$), and the 5% oxygen group (ANOVA, df = 2, 6, F = 33.476, $p<0.005$) (Figure 8). The tube scaffold tissue cultures had significantly lower DNA content compared to the construct tissue cultures under all oxygen tensions (all Bonferroni $p<0.05$). The tube scaffold tissue cultures also had significantly lower DNA content compared to the scaffold cultures in the 5% oxygen group (Bonferroni $p<0.05$). However, no significant differences were observed between these scaffold types in the "21% to 5% O_2" group and the 21% oxygen group (all Bonferroni $p>0.05$).

Thus, the tube cultures had significantly lower DNA content comparing to the construct under all oxygen tensions, and had significantly lower DNA content compared to the scaffold group under low oxygen tension. The tube tissue cultures had nearly 0 ng/ml for the "21% to 5% O_2" and 21% O_2 groups, which indicates that few, if any, cells were present on the scaffold (Figure 8).

5.2 CD31 Immunohistochemistry of scaffold tissue cultures under various oxygen tensions

Histology with CD31 immunohistochemistry was performed on the scaffold tissue cultures to qualitatively analyze cell presence in the tissue cultures and to analyze cell organization (Figure 9). Black dots represent CD31 antibody-stained cells (white arrow), whereas the larger, more lightly colored fragments are silk scaffold (red arrow). Considerably lower cell density was observed in the 21% oxygen tissue cultures as compared to the 5% oxygen tissue cultures and the "21% to 5% O_2" cultures. Little difference in cell density was observed between the 5% oxygen and the "21% to 5% O_2" cultures. No cellular organization resembling lumen formation was observed in the histology.

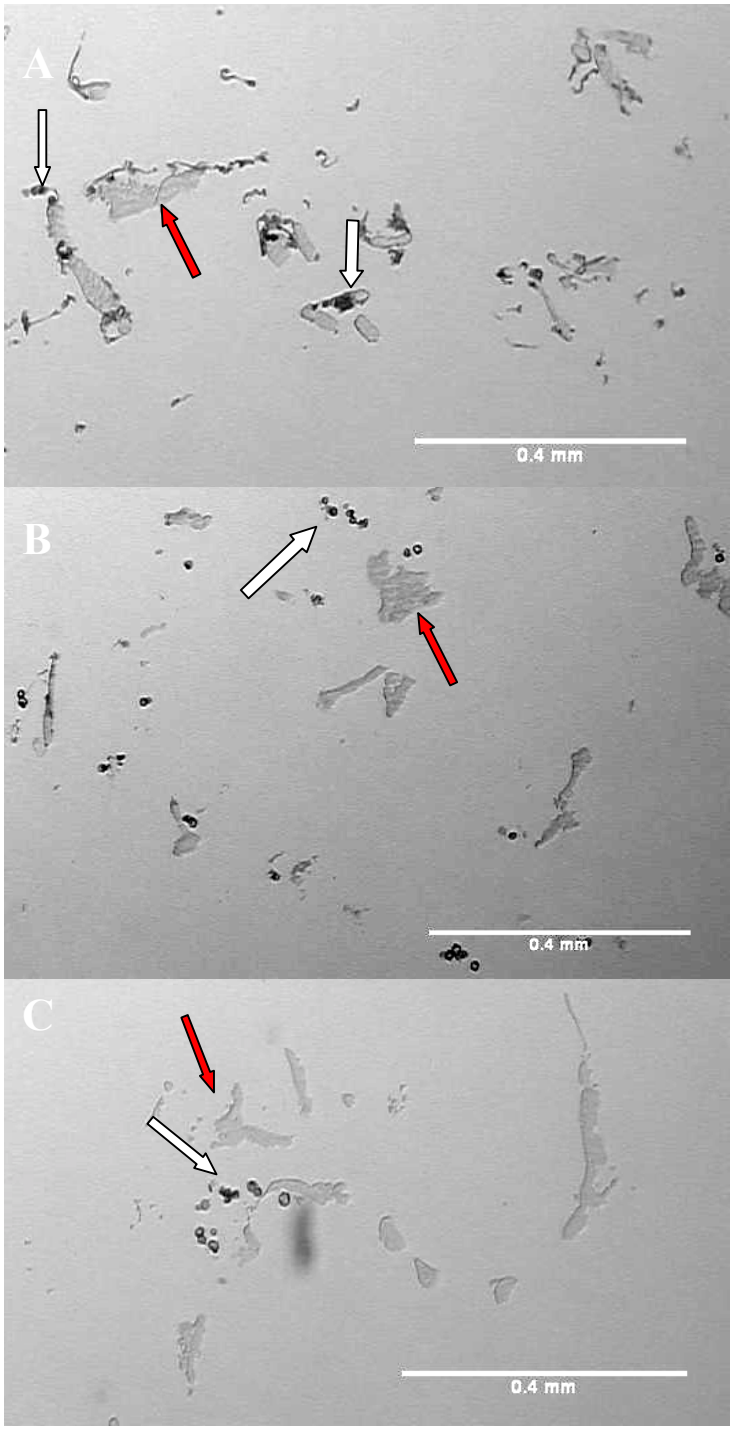

Figure 9: CD31 immunohistochemistry results of 2 week cultures in various oxygen tensions. **A**: 5% oxygen, **B**: 21% to 5% oxygen, **C**: 21% oxygen. White arrows indicate cells, and red arrows indicate scaffolds.

5.3 DNA quantification for tissue cultures under dynamic culture or under static conditions in various oxygen conditions

A DNA quantification assay was performed to determine (1) if oxygen tension affected cell number in tissue cultures exposed to shear stress, and (2) if oxygen tension affected cell number in static tissue cultures. DNA quantification assays were performed for tissue cultures grown under a gradual increased shear stress or under static conditions in various oxygen tensions (5% O_2 or 21% O_2) for 14 days.

Preliminary results showed that a surface shear stress of 9 dynes/cm^2 dramatically decreased cellular attachment to the scaffolds. HMVECs were seeded on a scaffold, allowed to attach for 24 hours, exposed to stepwise increases of shear stress (1.5 dynes/cm^2 per day) reaching 9 dynes/cm^2 after 7 days, and cultured for an additional 7 days, all under 21% oxygen. DNA quantification after 14 days culture showed that there were five fold less cells in dynamic culture samples as compared to static culture samples (results not shown). These results suggest that a surface shear stress of 9 dynes/cm^2 may be too high to feasibly culture cells in our system. Thus, in the following study, we reduced the surface shear stress to 4 dynes/cm^2.

In the subsequent study, the tissue cultures were cultured under static conditions or under dynamic culture (a stepwise increase in shear stress for 4 days, reaching a maximum of 4 dynes/cm^2) for 14 days in various oxygen tensions. A DNA content assay of the tissue cultures was performed at day 14, and the DNA content was normalized to day 0 seeding controls (Figure 10). No significant differences in normalized DNA content were observed between oxygen tensions in static conditions (Independent Student's t-test, df = 6, t= -2.095, $p > 0.05$). Also, no significant differences in DNA content were observed between oxygen tensions in dynamic culture (Independent Student's t-test, df = 6, t = -0.927, $p > 0.05$).

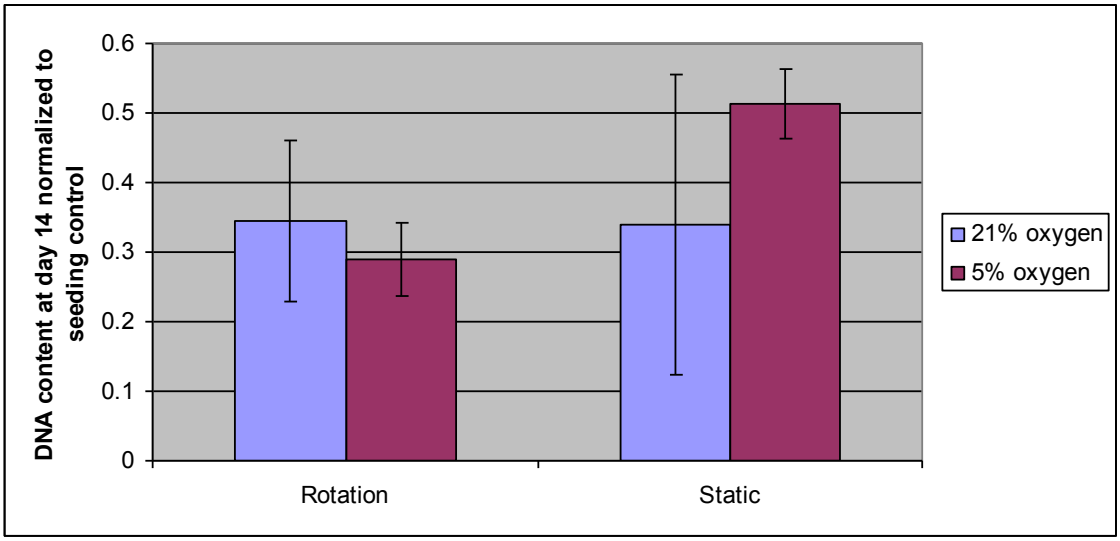

Figure 10: DNA quantification assay on dynamic (rotation) and static cultures in different oxygen environments. N=4 for all groups; error bars represent standard deviation. No significant differences were found between oxygen tensions in rotation culture or static culture (Independent Student's t-test, all $p > 0.05$).

Histology and H&E staining were performed on day 14 samples to qualitatively analyze cell number and cell organization. However, after sectioning, processing artifacts made it difficult to see any cells after staining (results not shown).

5.4 Quantification of CD31 (PECAM-1) expression in HMVECs in 21% oxygen or 5% oxygen and in static or dynamic culture.

Quantitative RT-PCR was performed on CD31 to determine if low oxygen conditions affected CD31 expression. Increased CD31 levels may indicate angiogenic activity (Park et al. 2010) and could explain increased cell retention under shear stress (Zhao et al. 2008). CD31 expression in HMVECs grown in static or rotation culture under various oxygen conditions was quantified by qRT-PCR (Figure 11). No statistically significant trends were observed between 21% oxygen groups and 5% oxygen groups, nor between static and dynamic culture conditions (ANOVA, $df = 5, 12$, $F = 3.056$, $p > 0.05$).

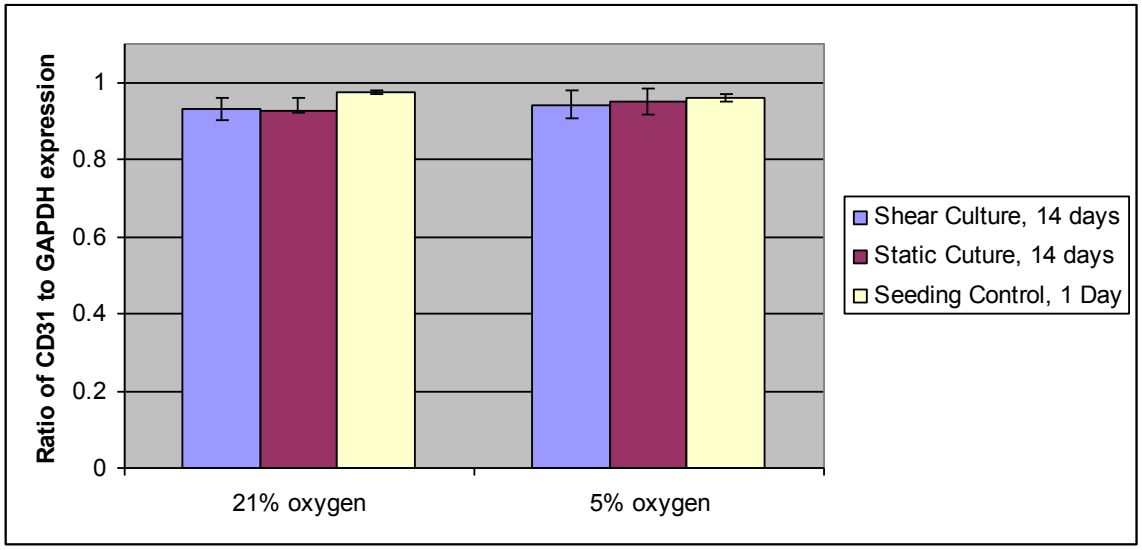

Figure 11: Quantification of CD31 expression in 21% and 5% oxygen conditions by qRT-PCR. Expression of CD-31 was normalized to GAPDH. Error bars represent standard deviations (n=3). No significant trends were observed.

5.5 DNA content assays of cells grown in 5% oxygen vs. 21% oxygen at various time points

A DNA content assay was performed to analyze cell number and to assess cell proliferation under various oxygen tensions. In this experiment, HMVECs were cultured in 21% oxygen or 5% oxygen, seeded in 3D porous silk scaffolds, and grown in tissue culture (under 21% oxygen or 5% oxygen) for 7 days with time points at 0 days (seeding control) and 3 days.

DNA content was measured after 3 and 7 days of culture and normalized to seeding controls (Figure 12). Statistically significant differences in DNA concentrations (normalized to the seeding control) were found between the 5% O_2, 21% O_2, and "21% to 5% oxygen" groups (ANOVA, df = 2, 12, F=12.17, $p<0.05$). DNA concentration of the 5% oxygen group was significantly lower than the 21% oxygen and "21% to 5%" oxygen groups (Bonferroni, $p<0.05$), while no significant differences were found between the 21% O_2 and "21% to 5% O_2" groups (Bonferroni, $p> 0.05$). No significant differences were observed between any of

the groups in day 7 (ANOVA, df = 2, 12, F= 1.459, p>0.05). This data shows that cell number was not markedly altered by oxygen tension in the 7 day culture.

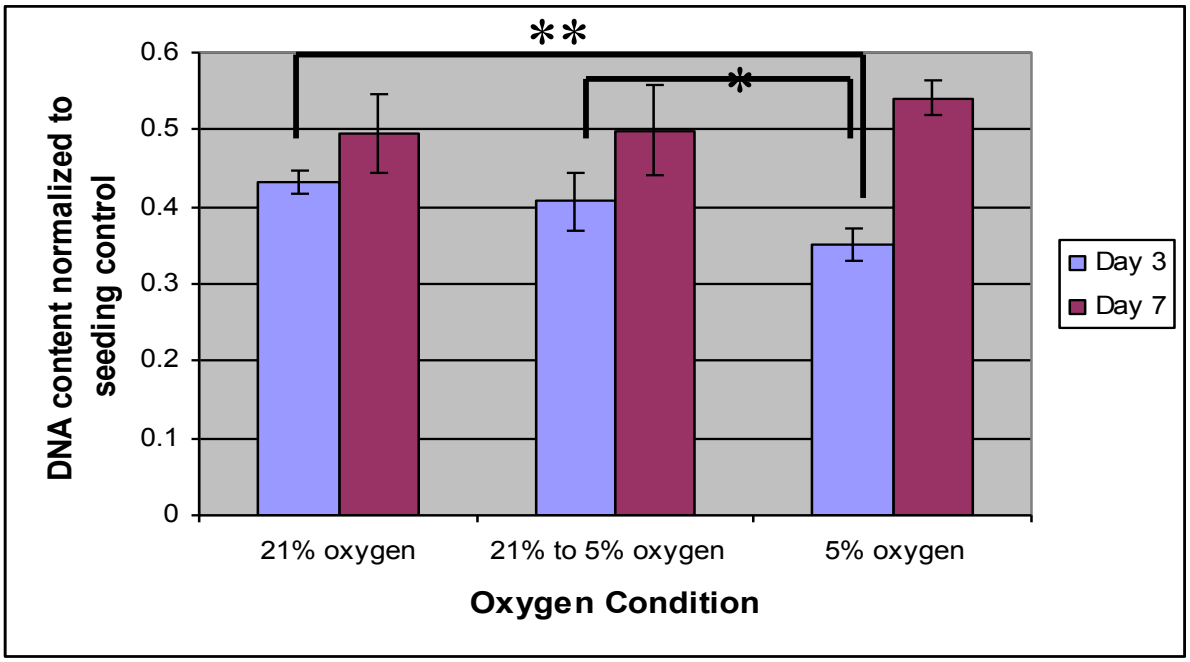

Figure 12: Normalized DNA content of tissue cultures in various oxygen tensions after 3 and 7 days in culture. DNA content was normalized to DNA content of the seeding control. Error bars represent standard deviation (N=5). At day 3, the 5% oxygen group had significantly lower DNA content compared to the 21% oxygen group (p**<0.05) and the 21% to 5% oxygen group (p*<0.05). No significant differences were observed at day 7 (all p>0.05).

We analyzed cell proliferation under various oxygen tensions by calculating the percent increase of DNA content from day 3 to day 7 (Figure 13). Statistically significant differences in percent increase of DNA content were observed between oxygen groups (ANOVA df = 2, 12, F = 16.955, p< 0.005). As can be seen in Figure 13, the 5% oxygen group had a significantly higher percentage increase in DNA concentration from day 3 to day 7 as compared to the 21% oxygen group (Bonferroni $p^{*} < 0.005$) and the "21% to 5% oxygen" group (Bonferroni $p^{**} < 0.005$), suggesting that the proliferation rate was higher for the 5% group compared to the other groups. However, there was no significant difference in

the percent increase of DNA concentration between the 21% oxygen group and the "21% to 5%" oxygen group (Bonferroni, p>0.05), which suggests that oxygen tension did not affect proliferation.

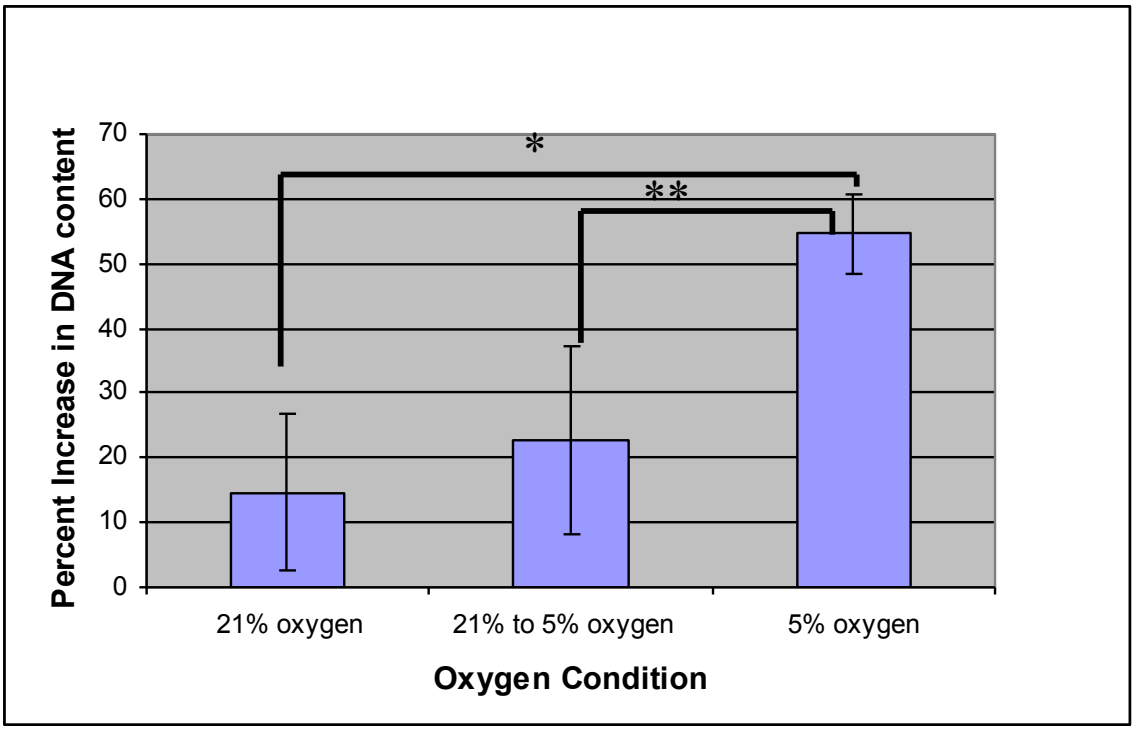

Figure 13: Percent increase in DNA content of HMVEC monocultures grown on a 3D scaffold from day 3 to day 7. Error bars represent standard deviations (N=5). Percent increase in DNA content was higher in the 5% oxygen group as compared to the 21% oxygen group ($p<0.05$) and the 21% to 5% oxygen group ($p<0.05$). No significant differences were found between the 21% oxygen group and the 21% to 5% oxygen group ($p>0.05$).

5.6 Confocal microscopy

Confocal microscopy was performed to qualitatively analyze cell number and to analyze cell organization of tissue cultures in 21% oxygen and 5% oxygen. Fluorescent images of DiD stained cells were taken at days 3 and 7 of the tissue cultures at various oxygen tensions. The DiD staining alone was not sufficient to obtain useful images the cells at day 3 (results not shown), so an additional nuclear stain (Vybrant DyeCycle Ruby stain) was added to the tissue culture samples at day 7 (Figure 14).

The Vybrant nuclear stain dyed the cells, but it also strongly stained the silk scaffold (representative images in Figure 14). While the staining of the silk made it difficult to

distinguish between cells and scaffold, we postulate that the "dots" in the figure represent cells (red circles indicate high cell number) while the green background represents scaffold (arrows in Figure 14). There was no observable cell organization (such as lumen formation) in the 21% oxygen group and the 5% oxygen group. However, there appeared to be higher cell density in the 5% oxygen group compared to the 21% group.

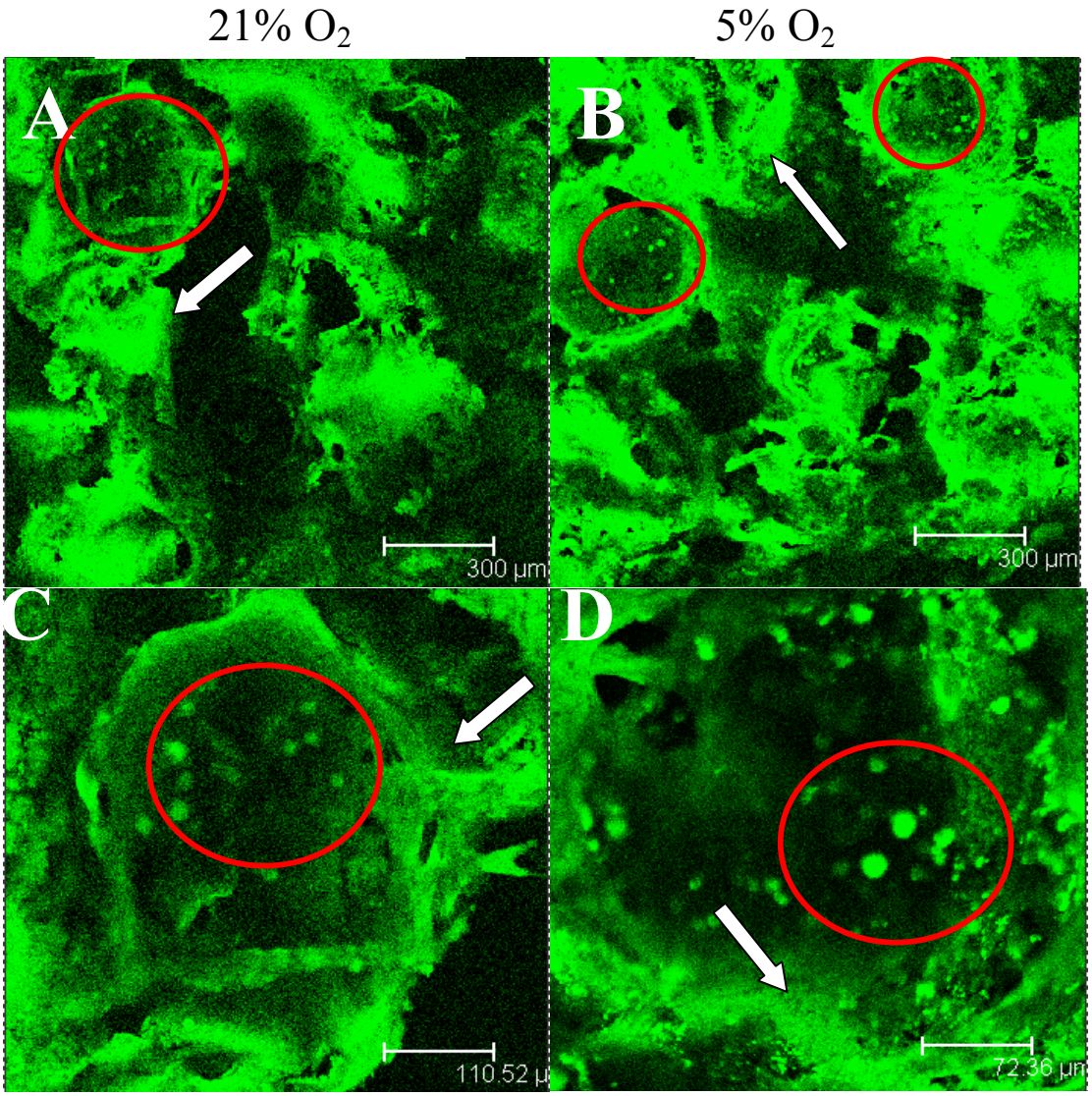

Figure 14: Confocal images of DiD/DyeCycle Ruby dye stained endothelial cell tissue cultures grown for 7 days at 5% O_2 (A, C) or 21% O_2 (B, D). Red circles highlight clusters of high cell density. White arrows indicate the stained scaffold.

Chapter 6: Discussion:

This study assessed the use of low oxygen (5% O_2) for culturing 3D vascular tissues. Recent studies have shown that culturing endothelial cells (HUVECs and HMVECs) in 5% oxygen in the presence of growth factors stimulates cell proliferation, endothelial cell organization, synthesis of ECM proteins such as fibronectin and synthesis of ECM binding proteins such as PECAM-1 (Zhou et al. 2000, Decaris et al. 2009, Zhao et al. 2008). Moreover, pre-treating endothelial cells with 5% oxygen has been shown to improve cell retention on PET films in high shear stress environments (Zhao et al. 2008). While these studies have shown the use of 5% oxygen for endothelial cell culture, they have not assessed the use of low oxygen treatments in 3D cultures.

In this study, we applied the use of low oxygen endothelial cell culture in 3D porous silk scaffold, with the goal to improve cellular proliferation and cell organization. Moreover we investigated the use of 5% oxygen culture for improving cell retention on 3D silk scaffolds in dynamic cultures. While some of our results suggest that 5% oxygen tissue culture may induce endothelial proliferation in 3D scaffolds, repeated iterations of the tissue culture provided conflicting results, making it difficult to evaluate 5% oxygen tissue culture, both in static and dynamic culture.

6.1 DNA Quantification of silk construct, scaffold, and tube tissue cultures grown under various oxygen tensions

In our preliminary experiment, endothelial cell tissue cultures, each seeded with 0.75 x 10^6 cells, were grown in a silk tube-scaffold construct, silk tube, or a silk scaffold under various oxygen tensions (5% oxygen or 21% oxygen) for two weeks. A DNA quantification assay was used to quantify cell presence in these tissue cultures (Figure 8). The results indicated that, in tube-scaffold constructs and scaffolds, 5% oxygen tissue cultures had

significantly higher DNA content as compared to 21% oxygen tissue cultures and 5% oxygen tissue cultures that were pre-cultured in 21% oxygen in 3D. This higher DNA content shows that the 5% oxygen group had a higher cell number than the 21% oxygen group. Also, the "21% to 5% oxygen" group cultured in tube-scaffold constructs had significantly higher DNA content compared to the 21% oxygen group cultured in constructs. These data agree with previous studies that showed increased cell proliferation of HUVECs under 5% oxygen conditions, as compared to 21% oxygen controls (Zhao et al. 2008). However, these results were inconsistent with the finding that the "21% to 5% oxygen" group cultured in silk scaffolds did not have significantly higher DNA content compared to the 21% oxygen group.

These results generally showed that 5% oxygen tissue cultures had more cells than 21% oxygen tissue cultures. In the scaffold only group, there was a 13 fold higher DNA content in 5% oxygen tissue cultures compared to the 21% group, and in the construct group there was a 6.5 fold higher DNA content in the 5% group compared to the 21% group (Figure 8). While this increase in cell number was expected, it is possible that seeding density was a confounding factor that produced systematic error in our results because, although the cells were counted in each group prior to seeding, there may have been errors in cell counting. In subsequent experiments, we included a seeding control group for DNA quantification experiments to normalize for cell counting errors.

Whereas significant differences were found between oxygen tensions in the construct and scaffold only endothelial cell tissue cultures, no significant differences were found between oxygen tensions in the silk tube group (Figure 8). These results were surprising, as marked differences were found between oxygen groups in different scaffolds. The DNA content was nearly 0 ng/ml for the 21% oxygen group and the 5% tissue culture group pre-

cultured in 21% oxygen, and the 5% oxygen group was 1.5 $\pm$ 0.75 ng/mL (Figure 8). These data indicate that there were few if any cells attached to the silk tube for the 21% oxygen and the "21% to 5%" oxygen group, and that there was a small cell number in the low oxygen group (as compared to the other scaffolds). Thus, before pursuing the development of this tube-scaffold construct, we must improve techniques for seeding and culturing endothelial cells on tube scaffolds to improve cellular attachment.

6.2 CD31 Immunohistochemistry of scaffold tissue cultures under various oxygen tensions

In addition to DNA quantification techniques, histology with CD31 immunohistochemistry and H&E staining were performed to qualitatively analyze cell presence in the tissue cultures and to analyze cell organization (Figure 9). In the scaffold only tissue cultures, lower cell numbers were observed in the 21% oxygen group as compared to the 5% oxygen group and the "21% to 5% group." These results confirm the DNA quantification results, which showed lower DNA content in the 21% oxygen group compared to the 5% oxygen group. Similar cell numbers were observed between the 5% oxygen group and the "21% to 5% oxygen" group. This result differs from the DNA quantification results, which showed lower DNA content in the "21% to 5% oxygen" group as compared to the 5% oxygen group. A possible explanation from this contradiction is that the sections observed may have been from different locations in the scaffold. However, it is difficult to conclusively explain this contradiction.

6.3 DNA quantification for tissue cultures under shear stress or under static conditions in various oxygen conditions

In our second study, we explored the use of 5% oxygen tension for increasing cell retention in dynamic 3D HMVEC tissue cultures. DNA content, normalized to seeding density, was used to quantify cell presence in 14-day tissue cultures exposed to shear stress

(gradually increased by 1 dyne/cm^2 per day reaching a max shear stress 4 dynes/cm^2) or grown in static conditions (Figure 10). No significant differences were found between oxygen groups in the dynamic cultures or in the static cultures.

It is important to note that there was a high standard deviation in both the static culture and the dynamic culture groups cultured in 21% O_2 conditions. Repeating these experiments with a higher sample size would decrease the variance in samples, which would allow the detection of smaller differences in DNA content between oxygen groups.

6.4 Quantification of CD31 (PECAM-1) expression in HMVECs in 21% oxygen or 5% oxygen and in static or dynamic culture

Quantitative reverse transcriptase PCR was performed to analyze CD31 expression of HMVEC tissue cultures in 21% oxygen culture or 5% oxygen culture in static or dynamic culture. No significant differences were found between any groups (Figure 11). This result contradicts with the findings of Zhao et al. 2008 and Zhou et al. 2000, which showed higher CD31 protein content when HUVECs or HMVECs were cultured under 5% oxygen as compared to 21% oxygen (Zhao et al. 2008, Zhou et al. 2000).

6.5 DNA quantification for tissue cultures under shear stress or under static conditions in various oxygen conditions

To determine the effect of oxygen tension on cell proliferation in our 3D HMVEC tissue culture model, we analyzed the DNA content of the culture at 3 days and 7 days, normalized to seeding controls (Figure 12). We found that the 5% oxygen tissue culture group had significantly lower DNA content at day 3 as compared to the other groups (21% oxygen group and “21% to 5% oxygen” group), but no significant differences were seen in any of the groups at day 7. These results suggest that oxygen tension did not improve cell growth in tissue culture, contradicting the findings in our preliminary experiment.

We also analyzed the percent increase of DNA content from day 3 to day 7 to analyze proliferation (Figure 13). The 5% oxygen culture had significantly higher percent increase in DNA content from day 3 to day 7 as compared to the 21% oxygen group and the 5% oxygen group. However, no significant differences were found between the 21% oxygen group and the "21% to 5% oxygen" group. While the 5% oxygen group showed the expected increased proliferation rate, the lack of a difference between the 21% oxygen group and the "21% to 5%" oxygen group was unexpected, as previous findings (Zhao et al. 2008) showed increased endothelial cell proliferation within two days of exposure to 5% oxygen as compared to 21% oxygen controls.

6.6 Confocal microscopy

Confocal microscopy was used to qualitatively analyze cell presence and to look for cell organization (lumen formation) in the samples (Figure 14). We found that the 5% oxygen group had more "pockets" of cells, and a higher density of cells as compared to 21% oxygen samples (representative images Figure 14). No lumen formation was observed in either sample, so we conclude that oxygen tension did not improve cell organization in our system. In both oxygen tensions, we observed higher cell density at the top of the scaffold (where the cells were initially seeded) as compared to bottom of the scaffold, which suggests that the scaffold was not uniformly seeded seeded. Different cell labeling mechanisms, such as GFP-transfection (as was used by Kang et al. 2009) could produce more reliable results.

6.7 HIF-1α western blot

One major concern with using 5% oxygen tension in our 3D tissue culture system was inadequate oxygenation of cells in the center of the scaffold. Inadequate oxygenation at the centers of large tissue cultures has been a major challenge in engineering thick tissues, as

diffusion limits are generally limited to 200 μm in tissues (Lovett et al. 2009). In our model, we made the assumption that these diffusion limits were not a problem because of (1) the small dimensions of our scaffold (4x 8 mm cylinder) (2) the high porosity of the silk scaffold and (3) we assumed the short term culture of endothelial cells would not create a thick tissue, and would therefore allow sufficient diffusion of oxygen. These assumptions were made because of the similarity of our procedures to Kang et al. 2009, which reported successful vascularization of engineered adipose tissue. However, the 5% oxygen culture would result in lower oxygen saturation in the media compared to 21% oxygen culture, which may lead to insufficient oxygenation of the core of the scaffold.

We performed a HIF-1α western blot to determine if cells in the 5% tissue cultures were experiencing hypoxic conditions compared to the 2D 5% oxygen cultures and the 3D 21% O_2 tissue cultures. However, we were unable to detect any HIF-1α protein or even the GAPDH control. This difficulty may have been due to low protein content in the tissue culture protein extracts.

Chapter 7: Future Directions:

Future studies need to be performed to further assess the effect of low oxygen (5%) culture on cell proliferation in static tissue cultures. While this study provided some evidence that low oxygen increased cell proliferation in static cultures, the results were somewhat contradictory. To improve our ability to assess proliferation in static tissue cultures, more time points should be included to improve the time course of the tissue cultures. Using this data, not only can the effect of low oxygen on proliferation be determined, but the time course of culture could be optimized as well. The timescale of the culture would be particularly important because high cell density could lead to oxygen diffusion problems, and culturing cells for too long in a low oxygen environment could lead to prolonged hypoxia in the center of scaffold, leading to cell death. Thus, if low oxygen treatment improves cell proliferation over a limited time scale, the oxygen environment of the cultures could be altered when insufficient oxygenation becomes a problem.

Moreover, tissue culture in different low oxygen environments (for example a range of 5-13% oxygen) may be used to optimize proliferation in this system. This study focused on the use of 5% oxygen culture because this oxygen tension was well studied (Zhao et al. 2008, Zhou et al. 2000, Decaris et al. 2009). However, Decaris et al. demonstrated that HMVEC proliferation was highest between 8 and 12% oxygen in 2D culture (Decaris et al. 2009). The use of oxygen treatment in this range could improve endothelial cell proliferation, and it may reduce hypoxia in the center of the tissue culture, as the dissolved oxygen content in the media would be increased.

If low oxygen tissue culture is pursued, the problem of oxygen diffusion to the core of the scaffold should be assessed. While the HIF-1α western blot may provide some

information about hypoxia in the scaffold, this technique may be limited because only a small number of cells may undergo hypoxia, and the signal may be undetectable. Thus, new techniques should be employed to more accurately determine oxygen diffusion problems in this system.

Perfusion systems could be engineered to overcome the problem of oxygen diffusion. A bioreactor system with perfusion through the center of the tissue culture could minimize oxygen gradients, allowing for a controlled cellular growth environment. Perfusion bioreactors have been used in engineering 3D bone tissue (Grayson et al. 2008) and vascular tissue models (Frerich et al. 2009). The use of scaffold with microchannels provides a straightforward approach for perfusion of 3D scaffolds (Lovett et al. 2009). Thus, these bioreactor systems could be used to create a controlled oxygen environment (mimicking *in vivo* oxygen concentrations) in larger 3D vascularized tissue constructs.

Future work should be done to assess the efficacy of low oxygen tissue culture on endothelial cell retention on silk scaffolds under shear stress. While low oxygen pre-culture has been shown to significantly increase cell retention on PET films under shear stress (Zhao et al. 2009), we did not observe increased cell retention on silk scaffolds in low oxygen groups in our studies. However, study of low oxygen pre-treatment on perfused tube scaffold tissue cultures could potentially improve cellular retention in these cultures, which can be used for tissue engineered vascular grafts.

Future work should also examine the use of gradual increases in shear stress to improve cellular retention to silk scaffolds in dynamic cultures. The use of gradual increases in shear stress has been successfully used to improve endothelial cell retention on vascular graft tissue cultures under dynamic conditions, and has been especially effective under high

shear stress (Inoguchi 2007, Ott and Ballerman 1995). While this technique was used in our model, only one sequence of gradual increase of shear stress was examined. Future studies should optimize these gradual increases in dynamic culture to improve endothelial cell retention on scaffolds. Combining the techniques of gradual increases in shear stress and low oxygen cell culture could provide an effective means of maximizing endothelial cell retention in dynamic cultures.

As the ultimate goal in these experiments is to create a vascularized tissue, these low oxygen-treated endothelial cell tissue cultures should be used in co-culture studies. These studies would truly assess the feasibility of low oxygen pre-cultured vascular platforms for tissue engineering.

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