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Angiogenesis And Neo-Microcirculatory Function In Diseased Tissue Revealed By Intravital Microscopy

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Graduate Program in Medical Biophysics

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ABSTRACT

Angiogenesis is the process of generating new blood microvessels. In adults, angiogenesis is fundamental to tumor biology and also to tissues rendered ischemic from vascular occlusion. Despite a promising appeal, strategies designed to simulate or otherwise modify the angiogenic process in adult tissues have yet to realize significant therapeutic potential. Importantly, understanding the structure and function of new microvascular networks formed in adult, diseased tissues is limited. In fact, it remains unknown if a new or regenerated microcirculation can effectively deliver oxygen to the tissue. The purpose of this thesis was to seek out novel determinants of functional angiogenesis and microvascular regeneration.

Using a novel real-time microscopy strategy for imaging red blood cells (RBCs), I interrogated microcirculatory architecture and perfusion in mouse renal cell carcinomas. I found that the tumor microvasculature was devoid of hierarchy, microvessel specification, and vasoreactivity. Furthermore, I found that fibroblast growth factor 9 can productively reconfigure the tumor microvasculature into a hierarchical network of arteriole-capillary-venular units, with vasomotor competence. Together, these findings revealed important microvascular abnormalities in tumors and established that attainment of a physiologically advanced microcirculation in tumors is achievable.

I next interrogated the structure and function of the regenerated microvasculature that forms in skeletal muscle following ischemic injury. I discovered that despite extensive angiogenesis, there are profound flaws in microvascular network geometry, hierarchy, RBC transit, flow control by hypoxia, and smooth muscle cell wrapping around arterioles. Thus, the neo-microvasculature in regenerated skeletal muscle does not recapitulate the anatomy and physiology of a normal microvasculature and is ineffective at controlling the delivery of oxygen.

Finally, I examined the early-stage events by which new microvascular networks form following skeletal muscle infarction. I discovered that vascular regeneration entails the abrupt appearance of metastable primordial microvessels with ultra-low blood flow.
further discovered that primordial microvessels rapidly transform into an extensive microvasculature via intraluminal endothelial cell protrusion and intussusceptive angiogenesis. This non-sprouting angiogenesis cascade was determined by differential endothelial cell VEGFR2 activation.

In summary, this thesis provides novel insights into adult angiogenesis and the workings of microvascular systems that have been injured by disease. These findings have relevance to strategies for restoring oxygen content in pathological tissues.

**Keywords:** angiogenesis, microcirculation, ischemia, intravital microscopy, skeletal muscle, cancer, microcirculatory function, endothelial cell, VEGFR2, intussusceptive angiogenesis
CO-AUTHORSHIP STATEMENT

All chapters of this thesis were written by Giovanni-Michele Arpino and further revised with recommendations from Dr. J Geoffrey Pickering. Portions of this thesis have been published and the contributions of the authors of these articles are as follows:


**Chapter 3** is based on original research published as: Arpino, J. M., Nong, Z., Li, F., Yin, H., Ghonaim, N. W., Milkovich, S., Balint, B., O'Neil, C., Fraser, G. M., Goldman, D., Ellis, C. G. & Pickering, J. G. 2017. “Four-Dimensional Microvascular Analysis Reveals that Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation.” *Circ Res*, 120, 1453-65. Giovanni-Michele Arpino designed and coordinated all experiments, performed all histology, immunostaining, intravital microscopy, and confocal microscopy experiments, and analyzed and interpreted all data. Zengxuan Nong and Fuyan Li performed mouse surgery and assisted with intravital microscopy experiments. Hao Yin and Brittany Balint performed quantitative real-time PCR. Nour Ghonaim, Stephanie Milkovich, and Christopher G. Ellis contributed to the design and analysis of the intravital microscopy experiments. Graham Fraser and Daniel Goldman contributed to the analysis of capillary network hemodynamic resistance. J. Geoffrey Pickering conceived and coordinated the study.
DEDICATION

To Nonno and Nonna
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<tbody>
<tr>
<td>$\alpha$</td>
<td>Alpha</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
</tr>
<tr>
<td>$\Delta \Delta CT$</td>
<td>Delta Delta Threshold Cycle</td>
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<tr>
<td>A</td>
<td>Arteriole</td>
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<td>ACV</td>
<td>Arteriole-Capillary-Venule</td>
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<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
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<tr>
<td>ANGPT1</td>
<td>Angiopoitin-1</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>AV</td>
<td>Arterio-Venous</td>
</tr>
<tr>
<td>AVI</td>
<td>Audio Video Interleaved</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BSI-B4</td>
<td>Lectin From Bandeiraea Simplicifolia</td>
</tr>
<tr>
<td>C</td>
<td>Capillary</td>
</tr>
<tr>
<td>$Ca^{2+}$</td>
<td>Calcium Ion</td>
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<td>$CaCl_2$</td>
<td>Calcium Chloride</td>
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<td>Chick Chorioallantoic Membrane</td>
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<td>Cdc42</td>
<td>Cell Division Control Protein 42</td>
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<td>Complementary Deoxyribonucleic Acid</td>
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<td>CLI</td>
<td>Critical Limb Ischemia</td>
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<td>CO$_2$</td>
<td>Carbon Dioxide</td>
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<tr>
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<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KLF2</td>
<td>Krüppel-like Factor 2</td>
</tr>
<tr>
<td>K-S</td>
<td>Kolmogorov-Smirnov</td>
</tr>
<tr>
<td>L</td>
<td>Length</td>
</tr>
<tr>
<td>LOXL2</td>
<td>Lysyl Oxidase</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MIN</td>
<td>Minimum</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mm³</td>
<td>Cubic Millimeter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MR</td>
<td>Magnetic Resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MV</td>
<td>Mother Vessel</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodium Phosphate Dibasic</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Monosodium Phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NG2</td>
<td>Neural Glial Antigen 2</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NRP1</td>
<td>Neuropilin-1</td>
</tr>
<tr>
<td>NRP2</td>
<td>Neuropilin-2</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical Cutting Temperature</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OPS</td>
<td>Orthogonal Polarization Spectral</td>
</tr>
<tr>
<td>P38 MAPK</td>
<td>P38 Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Arterial Disease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived Growth Factor</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet-derived Growth Factor Subunit B Homodimer</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet-derived Growth Factor Receptor β</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polymethylsiloxane</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>PEG-300</td>
<td>Polyethylene Glycol 300</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of Hydrogen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Peroneus Longus</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C Gamma</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental Growth Factor</td>
</tr>
<tr>
<td>PLOD2</td>
<td>Procollagen Lysyl Hydroxylase 2</td>
</tr>
<tr>
<td>pO2</td>
<td>Partial Pressure of Oxygen</td>
</tr>
<tr>
<td>PVD</td>
<td>Peripheral Vascular Disease</td>
</tr>
<tr>
<td>pVEGFR2</td>
<td>Phosphorylated Vascular Endothelial Growth Factor Receptor 2</td>
</tr>
<tr>
<td>Q&lt;sub&gt;tot&lt;/sub&gt;</td>
<td>Total Flow</td>
</tr>
<tr>
<td>R</td>
<td>Resistance</td>
</tr>
<tr>
<td>R&lt;sub&gt;norm&lt;/sub&gt;</td>
<td>Normalized Resistance</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 Botulinum Toxin Substrate</td>
</tr>
<tr>
<td>RBB</td>
<td>Retinal Blocking Buffer</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>Renca</td>
<td>Renal Cell Carcinoma</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-Phosphate</td>
</tr>
<tr>
<td>SAD</td>
<td>Sum of All Differences</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>Shc</td>
<td>Shc Adaptor Protein</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>SM</td>
<td>Smooth Muscle</td>
</tr>
<tr>
<td>SM-α-actin</td>
<td>Smooth Muscle Alpha Actin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium Nitroprusside</td>
</tr>
<tr>
<td>SO₂</td>
<td>Saturated Oxygen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>Sos</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>Sox18</td>
<td>Transcription Factor Sox18</td>
</tr>
<tr>
<td>Src</td>
<td>Proto-Oncogene Tyrosine-Protein Kinase</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis Anterior</td>
</tr>
<tr>
<td>Tie-1</td>
<td>Tyrosine Kinase with Immunoglobin-like and EGF-like Domains 1</td>
</tr>
<tr>
<td>Tie-2</td>
<td>Tyrosine Kinase with Immunoglobin-like and EGF-like Domains 2</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µm²</td>
<td>Square Micrometer</td>
</tr>
<tr>
<td>V</td>
<td>Venule</td>
</tr>
<tr>
<td>VE</td>
<td>Vascular Endothelial</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Vascular Endothelial Cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular Endothelial Growth Factor A</td>
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<tr>
<td>VEGF-B</td>
<td>Vascular Endothelial Growth Factor B</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>Vascular Endothelial Growth Factor C</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>Vascular Endothelial Growth Factor D</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular Endothelial Growth Factor Receptor 2</td>
</tr>
<tr>
<td>VEGFR3</td>
<td>Vascular Endothelial Growth Factor Receptor 3</td>
</tr>
<tr>
<td>V&lt;sub&gt;Exp Max&lt;/sub&gt;</td>
<td>Maximum Red Blood Cell Velocity</td>
</tr>
<tr>
<td>V&lt;sub&gt;RBC&lt;/sub&gt;</td>
<td>Red Blood Cell Velocity</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>WPB</td>
<td>Weibel-Palade Body</td>
</tr>
<tr>
<td>XZ</td>
<td>X-Z Axes</td>
</tr>
<tr>
<td>YZ</td>
<td>Y-Z Axes</td>
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</table>
CHAPTER 1
General Introduction

Critical limb ischemia and myocardial infarction are major causes of morbidity and mortality. This is despite substantial advances in medical and surgical therapy for vascular diseases. Accordingly, there has been considerable interest in capitalizing on the natural, biological processes that underlie the formation of new blood vessels. Blood vessel formation has been extensively studied in the context of embryogenesis and organ development. However, new blood vessels can also be stimulated to form in adult tissues. This can occur in response to hypoxia, and as part of the repair and regeneration of damaged tissues.

The neovascularization response to ischemic injury includes the process of angiogenesis, whereby new microvessels form from pre-existing vessels within the tissue (Carmeliet, 2000). Angiogenesis can be stimulated by exogenous factors and, accordingly, there has been extensive effort to therapeutically enhance the process in ischemic tissues. However, although “therapeutic angiogenesis” strategies have appeared promising in animal models, carefully performed human studies have shown no benefit to the patient in terms of improving perfusion (Annex, 2013, Simons et al., 2003, Tongers et al., 2008).

For a new vascular network to function it must have the ability to exquisitely control blood flow and oxygen delivery in order to meet the local tissue needs, needs that vary in space and time. This matching process requires the formation of a hierarchical microvascular network, with vasoreactive arterioles, capillaries with single-file red blood cell transit, and draining venules. Whether such a network forms during angiogenesis in ischemic tissues is currently unknown. Moreover, the pathways by which a microvascular network forms in ischemic tissue in the adult are poorly understood.

A central premise of my thesis is that current understanding of adult angiogenesis is compromised by a lack of tools to investigate the regenerated microcirculation at a
network level, and with the required spatial and temporal resolution. Accordingly, the **broad goals of my thesis** were to develop strategies to interrogate the process of angiogenesis in adult tissues at a network level, and to utilize these strategies to determine i) the physiological attributes of a regenerated microcirculation, and ii) the steps by which that microvasculature forms.

To address these overarching goals, I have studied angiogenesis in two adult tissue environments: tumors and ischemic skeletal muscle.

**My specific objectives** were:

1. To develop high-resolution, real-time intravital microscopy-based strategies to elucidate the architecture and function of a newly formed microcirculation in diseased tissue, specifically renal tumors and injured skeletal muscle.

2. To determine the extent to which the architecture and function of the regenerated microcirculation in the mouse hindlimb recapitulates that of the normal muscle microcirculation.

3. To determine the architectural and cellular processes by which the early vascular network forms in regenerating skeletal muscle following ischemic injury.

## 1.1 Human Cardiovascular Disease

### 1.1.1 Ischemic Cardiovascular Disease

One of the greatest burdens of human disease and mortality globally is cardiovascular disease (Yusuf et al., 2001). Particularly devastating are the cardiovascular diseases that involve the narrowing or blockage of arterial vessels. This is because they can diminish or prevent blood flow into organs, which causes ischemic tissue injury and necrosis. This is manifested in human diseases including, but not limited to, atherosclerosis, peripheral arterial disease, coronary artery disease, hypertension, and stroke.
Feeder arteries are a particularly vulnerable component of the vasculature, and are the major type of vessel within the vascular tree affected by these diseases. Because of this, intensive preclinical research and human studies have been ongoing for decades, in order to unravel the cellular mechanisms regulating arterial health, disease, and repair. However, to successfully combat the clinical burden imparted by ischemic cardiovascular disease, the advancement of existing therapies and the development of new therapeutic strategies are required.

1.1.2 Peripheral Arterial Disease

Peripheral arterial disease (PAD), also known as peripheral vascular disease, is an ischemic cardiovascular disease affecting the legs, arms, and neck. PAD is caused by the development of systemic atherosclerosis. Atherosclerosis results in the narrowing and occlusion of feeder artery lumens, and restricts the supply of blood flow to the extremities (Annex, 2013). Restriction of blood flow induces ischemic injury in tissues downstream of the obstruction. In severe cases, total arterial blockage will result in marked tissue hypoxia and necrosis.

PAD is an important global health problem being the third leading cause of cardiovascular mortality, after coronary artery disease and stroke (Fowkes et al., 2013). The prevalence of PAD increases with age, affecting up to 20% of individuals over 70 years of age (Roger et al., 2011). Patients with diabetes are at a higher risk of developing PAD, and other causative risk factors for the development of PAD include hypertension, hypercholesterolemia, obesity, and smoking (Roger et al., 2011). The clinical manifestations of PAD include impaired limb function and reduced quality of life, as a consequence of reduced blood flow to the legs at rest or during exercise (Hankey et al., 2006).

Critical limb ischemia (CLI) is the most severe form of PAD. This is associated with pain at rest and can also be associated with skin ulcers and gangrene. Patients with CLI have a 25-40% risk of limb amputation and an annual mortality rate of 20% (Roger et al., 2011).
Peripheral human limbs are comprised mainly of skeletal muscle and thus muscle tissue is the most affected organ in PAD. Skeletal muscle is a metabolically active tissue composed of tightly arranged myofiber bundles that generate longitudinal contraction (Bach et al., 2004). As skeletal muscle requires a large amount of blood flow during both rest and exercise to support its metabolic activity, it is particularly vulnerable to ischemic injury and necrosis following arterial occlusion (Hurme et al., 1991). PAD restricts blood flow into expansive capillary networks embedded within skeletal muscle, and reduces tissue oxygen content.

1.2 The Microcirculation

1.2.1 Role of the Microcirculation

The microcirculation contains the smallest blood vessels within the cardiovascular system, ranging from 5 µm to ~150-200 µm in diameter (Levy et al., 2001). The main role of the microcirculation is to transport oxygen (O₂) and nutrients to every living cell of an organism through a series of interconnected tubes (Pries et al., 1995, Segal, 2005). The microcirculation is a highly organized tree-like network of arteries, arterioles, capillaries, venules, and veins. These microvessels are structurally arranged to optimize O₂ transport within a given organ.

Arteries and arterioles have a straight and slender morphology and are blood flow feeder channels. They control systemic vascular resistance and regulate the magnitude of O₂-rich blood flow into downstream capillaries (Segal, 2005). Capillaries are the smallest microvessels wherein RBCs transit in single file. Moreover, capillaries are the site of effective O₂ exchange from RBCs into the surrounding tissue, and are interconnected in distinct meshwork patterns that are specific to the host organ. Venules and veins are irregular-shaped and dilated channels with low blood pressure. They drain RBCs that have flowed through capillaries and have delivered their O₂ and return the RBCs to the heart and lungs for reoxygenation.

A functional microcirculation must exert dynamic control over tissue oxygenation, despite the short diffusion distance of O₂ from the blood into surrounding cells (Ellis et al., 2005). For this to occur, the microvasculature must be highly responsive
to dynamic changes in metabolic demand and adjust blood flow accordingly (Segal, 2005). This is crucial for preventing ischemia in tissues such as skeletal or cardiac muscle that have high metabolic requirements. If a microvasculature is dysfunctional and has an impaired ability to control flow, the tissue is rendered hypoxic as a result of a maldistribution of blood flow and O$_2$ delivery (Ellis et al., 2005). This is a consequence of diminished arterial vessel tone regulation via impaired communication between different segments of the microvascular tree (Segal, 2005, Ellis et al., 2005).

1.2.2 Cellular Anatomy of the Microcirculation

1.2.2.1 Arteries and Arterioles

The healthy artery in humans and mice is characterized by a three-layer multicellular arrangement (Lusis, 2000, Balint et al., 2015). The innermost layer is called the intima and is comprised of endothelium that makes direct contact with blood. The intima is separated from the second layer – the media, by a sheet of elastin fibers called the internal elastic lamina. The media is composed of one or more collectivized layers of vascular smooth muscle cells (SMCs), which contain contractile machinery and regulate vessel tone (Balint et al., 2015). The adventitial layer is the third and outermost layer and is comprised of loosely layered collagen, fibroblasts, immune cells, scattered SMCs, and progenitor cells (Passman et al., 2008). Small arteries and arterioles, including terminal or precapillary arterioles, are characterized by a two-layer arrangement. They are comprised of a monolayer of endothelial cells (ECs) that is tightly wrapped by one or two layers of SMCs (Figure 1.1)

The endothelium lines every blood vessel, not just arteries, and is composed exclusively of a monolayer of ECs (Sumpio et al., 2002, Ross, 1993) (Figure 1.1). ECs arise from a primordial vasculature that invades a developing organ in the embryo. ECs sustain normal microcirculatory function. This includes modulating vascular tone, controlling vessel permeability, and controlling immune responses by regulating the adherence of monocytes, platelets, and leukocytes to the inner vessel wall (Sumpio et al., 2002). The endothelium is also a paracrine and endocrine organ that influences the activity of ECs, perivascular cells, and circulating blood cells (Sumpio et al., 2002). ECs
**Figure 1.1 Anatomy of the microcirculation**

The microcirculation is comprised of a network of arteries, arterioles, capillaries, venules, and veins. Illustrated in this schematic is the cellular composition of the different vessel types in the microcirculation. Arteries are comprised of endothelial-lined channels that are fully wrapped by circumferential SMCs. As arteries branch into smaller arterioles and precapillary or terminal arterioles, the endothelium remains wrapped by SMCs but the wrapping orientation is altered. SMCs extend more longitudinally along the vessel and individual SMC processes circumferentially wrap the vessel. Capillaries are mainly comprised of ECs with scattered wrapping pericytes. Venules and veins are the largest endothelial tubes within the microcirculation and are invested by SMCs in a patchwork-like pattern.
Endothelial Cell
Smooth Muscle Cells
Pericyte
Red Blood Cell

Artery
Arteriole
Precapillary / Terminal Arteriole
Capillaries
Postcapillary Venule
Vein

Not to scale
form powerful adhesions with their neighbours with VE-Cadherin adherens junctions and tight junctions, in order to maintain vessel stability and form a tight barrier (Li et al., 2012). In disease or following injury, the EC monolayer becomes perturbed and the contact between neighbouring ECs is weakened.

SMCs and SMC processes circumferentially wrap the endothelium in arterial vessels (Figure 1.1). A circumferential arrangement provides structural integrity to the vessel, and allows SMCs to effectively constrict and relax in a coordinated manner to regulate vessel diameter (Clark et al., 1985). The embryological origins of SMCs vary depending on vessel size and location, but SMCs within the microvasculature appear to be derived locally within the organ parenchyma (Majesky, 2007). As a result, SMCs in different arteries may respond differently and have a spectrum of phenotypes. SMCs are identified by their expression of SM-α-actin and SM myosin heavy chain, among other markers (Owens et al., 2004). In some tissues such as bone, SMCs can express SM-α-actin and also the pericyte cell marker neural glial antigen 2 (NG2) (Kusumbe et al., 2016).

Although the circumferential wrapping pattern of SMCs is intrinsic to healthy arteries, SMCs can adopt specific orientations depending on their position within the arterial tree (Hill et al., 2015). There is a shift in SMC wrapping orientation from fully circumferential in arteries, to more parallel and spanning longer distances longitudinally as the vessel size decreases in arterioles near capillaries. However both orientations envelop the entire vessel circumference (Hill et al., 2015). In contrast to healthy arteries, disordered SMC wrapping arrangements characterize diseased arteries (Gown et al., 1986, Pickering et al., 1996). Furthermore, vasomotor competency and EC survival in arteries is not possible without the circumferential wrapping of ECs with SMCs (Hill et al., 2015).

1.2.2.2 Capillaries

Capillaries are 3-7 µm-diameter microvessels (Birbrair et al., 2014). They are positioned between arterioles and venules and have the thinnest walls of any vessel in the circulatory tree. Capillaries are comprised mainly of ECs, with scattered wrapping
pericyte cells (Birbrair et al., 2014) (Figure 1.1). ECs within capillaries are elongated and aligned to the direction of flow, and are positioned in series along the length of the microvessels. Most capillaries are completely continuous with tightly joined ECs, however some specialized capillaries contain fenestrations between ECs that enable a high level of vascular permeability.

Pericytes have recently become recognized as widespread components of the microvascular cellular architecture. By definition, they have short and thin cellular processes that project for hundreds of microns longitudinally and orthogonally to the vessel, but rarely wrap the entire vessel circumference (Hill et al., 2015). They make direct physical contact with ECs via longitudinal cytoplasmic processes that penetrate the EC basement membrane. Their abundance of coverage on the abluminal side of the capillary endothelium ranges from 10-50% depending on the organ (Armulik et al., 2005). In skeletal muscle, the pericyte to EC ratio has been estimated to be approximately 1:100, whereas in the retina the ratio is closer to 1:1 (Shepro et al., 1993).

Pericytes play critical roles in capillary development, stabilization, maturation, and remodeling (Armulik et al., 2005). Pericytes have been shown to have progenitor-like properties by having the ability to give rise to a variety of mesenchymal cell types (Farrington-Rock et al., 2004, Dellavalle et al., 2007, Brachvogel et al., 2005). Pericytes are also important for the tissue repair process and have been shown to participate in skeletal muscle regeneration and myogenesis (Birbrair et al., 2013). However, very little is known about their function in the skeletal muscle microcirculation. Pericytes are distinguished from other perivascular cell types by their expression of NG2, platelet-derived growth factor receptor β (PDGFRβ), desmin, and sometimes SM-α-actin (Lindblom et al., 2003). The fact that they express contractile elements including SM-α-actin indicates that they may be involved in regulating vessel tone. However, there is a substantial amount of controversy surrounding this idea. Recently, it was demonstrated in the brain microvasculature that pericytes do not regulate vessel diameter and blood flow (Hill et al., 2015). Whether this is the case for all microvascular beds has not fully been elucidated. This is partially because the distinction between pericyte and SMC morphology, surface markers, and location is not absolute. There is a continuum of
phenotypes ranging from the classical description of SMCs to that of pericytes, distributed along the entire vascular tree (Armulik et al., 2005, Herman et al., 1985).

1.2.2.3 Venules and Veins

Venules and veins are endothelium-lined conductance channels covered by pericytes and SMCs (Holley et al., 1983, Baumann et al., 1981). However, the SMC wrapping of venous vessels is less extensive and ordered compared to that in arterial vessels. The SMC wrapping pattern is circumferential and band-like, but individual cells cover larger surface areas of the vessel and exhibit a looser patchwork-like appearance (Hill et al., 2015) (Figure 1.1). While arteries and arterioles regularly dilate and contract, venules and small veins rarely change their diameter. Moreover, extensive distension of veins is a pathological feature of a diseased microvasculature (Raffetto et al., 2007)

1.2.3 Network Architecture and Hierarchy

The architecture of a microvascular network refers to the organization and patterning of individual microvessel segments within a tree-like arrangement. Normal network architecture is exquisitely organized in an artery-arteriole-capillary-venule-vein vessel hierarchy (Arpino et al., 2017) (Figure 1.1). A hierarchical architecture is essential for optimizing O$_2$ delivery to tissues.

The architecture of a microvascular network is pre-determined by genetic factors at birth and renders organ-specific patterns (Pries et al., 1995, Jones et al., 2006). Following the initial development of network architecture, further modification to network branching patterns and vessel caliber is induced by hemodynamic factors and local environmental signals (Pries et al., 1995, Jones et al., 2006).

Capillaries must be positioned between terminal arterioles and postcapillary venules in order to optimize O$_2$ delivery. Moreover, capillaries must be properly interconnected and dispersed in order to optimize the distribution of oxygenated RBCs from terminal arterioles into downstream individual capillaries (Ellis et al., 1994, Less et al., 1991, Pries et al., 1989). This also permits an intrinsic and diverse heterogeneity of RBC perfusion throughout individual capillaries that are fed by the same arteriole, which
is fundamental for tightly matching the delivery and distribution of blood flow to the localized requirements of tissues (Duling et al., 1987, Ellis et al., 1994).

1.2.3.1 Network Architecture in Skeletal Muscle

The skeletal muscle microvasculature is one of the most elegant examples of a normal and highly organized network architecture (Ellis et al., 1994, Arpino et al., 2017). Feeder arteries penetrate deep within the tissue, and arterioles and terminal arterioles symmetrically branch off from feeders at regular intervals and in orthogonal orientations to the muscle fibers. Each terminal arteriole branches into and supplies a dense meshwork of capillaries. Capillaries in skeletal muscle are aligned parallel to the muscle fibers and are 4 - 6 µm in diameter (Baum et al., 2013). They course for approximately 0.5 – 1 mm in length between individual muscle fibers, with many anastomoses between parallel segments (Emerson et al., 1997). Postcapillary venules are positioned between two terminal arterioles and collect blood from the parallel capillary beds. They dive into the tissue and drain blood into larger veins positioned deeper within the muscle.

In mouse skeletal muscle, the most distal arterioles (7-20 µm in diameter) have a single layer of circumferentially oriented SMCs, with some discontinuity in wrapping along the vessel length in the form of spaces between individual cells and processes (Holley et al., 1983). The entire SMC body spirals around the circumference of the vessel in 1-2 turns in larger caliber arterioles (Figure 1.1). However, in precapillary arterioles, SMCs extend over a longer distance with the nucleus oriented to the long axis of the vessel, and individual cell processes circumferentially wrap the vessel (Holley et al., 1983) (Figure 1.1).

In 1919, August Krogh first described the significance of the strategic positioning of capillaries between individual muscle fibers - to maximize the effectiveness of O2 delivery (Krogh, 1919). He generated a mathematical model that proposed that each capillary supplies O2 to a cylindrical volume of tissue surrounding the vessel segment. Furthermore, the model demonstrated that simply delivering O2 in bulk to an organ is ineffective, and that O2 must be distributed properly within the tissue to minimize O2-free zones. Krogh’s model highlights the impact of gas diffusion limitations on tissue.
oxygenation, and explains why the density of capillaries is elevated in tissues with high 
O₂ demand and consumption such as skeletal muscle.

Because of these physical principles, capillaries are widely distributed through the 
muscle tissue volume and every skeletal myocyte must be less than ~25 µm from a 
capillary O₂ supply (Ellis et al., 2012). Moreover, aerobic slow-twitch muscle fibers are 
surrounded by more capillaries compared to anaerobic fast-twitch muscle fibers (Kusters 
et al., 2016). It has recently been shown that capillaries are embedded within grooves of 
muscle fibers in order to maximize the contact surface area between myocytes and 
capillaries, and that mitochondria line these grooves in order to minimize the O₂ diffusion 
distance (Glancy et al., 2014).

**1.2.3.2 Network Architecture in Tumors**

Like all solid organs, tumors that have reached a threshold size will have their own 
microvasculature. However, the microvascular network architecture in tumors is 
abnormal and highly disordered. Tumor vessels are seemingly oriented at random within 
the tissue, and network branching patterns are haphazard (Yin et al., 2015). The diameter 
of the channels in tumors is notably wider than that of normal microvessels (Tong et al., 
2004, Less et al., 1991). Moreover, there are avascular and poorly vascularized regions 
scattered throughout tumors.

An organized microvascular network architecture is largely missing in the tumor 
avascularity (Yin et al., 2015). Instead, the network is characterized by disordered loops 
and meshworks (Tong et al., 2004, Jain, 1988, Less et al., 1991). Both asymmetrical 
arteriolar branching and supernumerary branching are also features of the disordered 
tumor microvasculature (Fukumura et al., 2010, Less et al., 1991).

Tumor vessel walls are sparsely covered by pericytes and SMCs and the specific 
wrapping orientation of these cells is not understood (Nagy et al., 2009, Fukumura et al., 
2010). Although growing tumours can surround and co-opt native arteries, the ability of 
vessels within the tumor to respond to vasomotor signals and regulate vessel tone has not
been identified. Together, these features of the aberrant tumor microvascular architecture render a hypoxic tissue environment (Helmlinger et al., 1997, Yin et al., 2015).

1.2.4 Molecular Specification and Hierarchical Differentiation of Vessels

The physiologic specification of vessels into arteries, capillaries, and veins is required for a vascular network to function properly. The endothelium does not consist of a globally homogeneous EC population, but instead a variety of molecules and pathways differentially mark the ECs that line arteries, capillaries, and veins. ECs express distinct arterio-venous (AV)-specific surface markers and proteins depending on their home vessel type and organ (Cines et al., 1998, Nunes et al., 2011).

In the embryo, distinct EC phenotypes contribute to the AV specification and differentiation of vessels. Embryonic ECs express different AV-specific markers even before the onset of a heartbeat or blood flow (Wang et al., 1998). This results in the formation of a hierarchical microvascular tree that develops from a primitive capillary plexus (Rocha et al., 2009). The first identified AV-specific markers were Ephrin-B2, which is expressed only in arterial ECs, and its receptor EphB4, which is expressed only in venous ECs (Wang et al., 1998). Since then, several pan-EC markers and specialized AV markers have been identified.

Pan-EC markers that are expressed by the endothelium in all types of vessels include cluster of differentiation 31 (CD31)/PECAM-1, VE-Cadherin, and vascular endothelial growth factor receptor 2 (VEGFR2) (Simons et al., 2015). Von Willebrand factor (vWF) is often considered to be a pan-EC marker but in fact is more strongly expressed in the endothelium of large vessels compared to microvessels (Kumar et al., 1987, Turner et al., 1987).

Arterial ECs specifically express the gap junction proteins Connexin-37 and Connexin-40, the transcription factor Sox18, the vascular endothelial growth factor (VEGF) co-receptor neuropilin-1 (NRP1), and EphrinB2 (Rocha et al., 2009). The crosstalk between Ephrin-B2 expressed by arterial ECs, and EphB4 expressed by mural cells is required for mural cell recruitment to arterial vessels (Korff et al., 2008). Venous
ECs express neuropilin-2 (NRP2), vascular endothelial growth factor receptor 3 (VEGFR3), and EphB4 (Rocha et al., 2009).

Interestingly, capillary ECs express a mosaic of the aforementioned AV-specific markers, depending on whether they are located at the arterial (upstream) or venous (downstream) end of a capillary bed. In addition, Dll4 and Notch are expressed mainly in capillary and arterial ECs (Shutter et al., 2000). Furthermore, the ECs in capillaries and veins express endomucin, but endomucin is not expressed in ECs in arteries (dela Paz et al., 2009). VEGFR2 is expressed more in the capillary ECs towards to the arterial end of a microvascular network, whereas VEGFR3 is restricted in the capillary ECs towards the venous end of the network (Rocha et al., 2009).

During adult neovascularization, AV specification is considerably plastic. ECs can change phenotypes during the generation and differentiation of new microvessels and microvascular networks (Nunes et al., 2011). This indicates that while AV specification is pre-determined in the embryo, it can be influenced by environmental cues during active vessel growth and remodeling.

Microvessels can also acquire organ-specific specialization in response to locally derived signals. This results in specific morphological and function features of the endothelium. In the intestine, liver, and kidney, capillaries contain small pore-like openings, called fenestrations, which allow high local permeability and rapid exchange of solutes between the blood and surrounding tissue (Stan et al., 2004). In contrast, in the central nervous system, the ECs must form a tight barrier so that trans-endothelial transport can be tightly regulated (Engelhardt, 2003).

1.3 Regulation of Blood Flow in the Microcirculation

The vascular tree does not act as an inert set of blood flow channels but, instead, as an intricately coordinated vasomotor system that controls the magnitude and distribution of blood flow into tissues as required (Segal, 2005). Vascular tone at the arterial level determines blood pressure and flow magnitude, and represents the interaction between the myogenic contraction of SMCs and the vasodilatory influence of
ECs (Segal, 2005). Several control systems regulate vascular tone throughout the circulation including neural impulses, circulating hormonal factors, local myogenic responses, and retrograde conducted vascular signaling (Segal, 2005, Clifford, 2011).

In order to tightly match microvascular blood flow with local requirements, vasomotor regulatory signals must be highly integrated throughout the entire microvasculature (Duling et al., 1987, Segal et al., 1989, Segal, 2005). The endothelium plays a pivotal role in this process. ECs integrate and conduct local chemical and mechanical stimulatory cues throughout vessel walls via electrical communication between ECs, and between ECs and SMCs (Segal et al., 1987, Segal et al., 1989, Dietrich et al., 1992, Koller et al., 1991).

Intriguingly, skeletal muscle has the largest capacity of any organ to alter arterial tone and microcirculatory blood flow, and can increase blood flow 100-fold as needed (Thomas et al., 2004).

1.3.1 Flow-Mediated and Conducted Vasomotor Responses

One of the important mediators of vascular tone is blood flow. Muscle activity stimulates an increase in blood flow and RBC velocity through arteries, which induces the enlargement of the vessel diameter (Segal, 2005). Arterial vasodilation in response to flow is mediated in part by the release of soluble vasodilatory agents such as nitric oxide (NO) and prostacyclin from stimulated ECs (Clifford, 2011). These agents instruct SMCs to relax, which locally decreases arterial tone and flow resistance. In turn, this increases blood flow and transmural pressure in proximal feeder arteries. Proximal arteries then consequently vasodilate and maintain the increased delivery of blood flow into distal arteries where the response originated.

In addition, locally initiated vasomotor responses can be conducted to distant arms of the arterial tree. This enables the vasculature to coordinate the magnitude and distribution of blood flow between separate arterial branches within vascular networks at the organ-level (Welsh et al., 1998). Moreover, conducted signaling determines whether vasodilation or vasoconstriction prevails. At high levels of metabolic demand, the locus...
of regulating blood flow shifts from dilated terminal arterioles to upstream resistance arteries in a process called conducted or ascending vasodilation (Hilton, 1959).

The mechanism controlling conducted vasomotor responses occurs via the spread of electricity throughout the walls of blood vessels. This response can initiate in capillaries and venules before spreading to arteries. The electrical signal originates in the endothelium, wherein charged ions travel rapidly through gap junctions (Segal, 2005). When endothelial cells are stimulated, they become hyperpolarized and internally release Ca$^{2+}$. Ca$^{2+}$ spreads through gap junctions between neighbouring ECs and this promotes the hyperpolarization of SMCs, via the production of hormones that stimulate SMC K$^+$ efflux and relaxation. As well, the activation of K$^+$ channels in ECs can hyperpolarize and relax SMCs, via electrical coupling through heterocellular myoendothelial coupling (Welsh et al., 1998, Emerson et al., 2000). An influx and spread of intracellular Ca$^{2+}$ and the activation of α-adrenoreceptors within SMCs mediates SMC contraction (Segal, 2005).

Therefore, electrical conduction through vascular cell gap junctions provides an efficient and rapid pathway for coordinating vasomotor tone amongst different branching orders of the arterial tree. Impaired endothelial conduction likely occurs in a variety of diseases including diabetes and sepsis, which are associated with a compromised control of vasomotor tone (Bateman et al., 2008, Frisbee, 2005).

1.3.2 Blood Flow Shear Stress and Mechanotransduction

For ECs to properly conduct and transmit vasomotor signals, they require an intrinsic ability to directly sense blood flow. Specifically, ECs can sense blood flow shear stress and transmit the signal to the cell interior (Ando et al., 2009, Traub et al., 1998, Resnick et al., 2003). The transmission of blood flow shear stress forces into the EC interior is known as mechanotransduction.

Shear stress is defined as the dragging frictional force imparted by blood on the endothelium in the direction of flow (Al-Khazraji et al., 2016). Shear stress is the product of blood viscosity and vessel wall shear rate, the latter being the tangential flow velocity
gradient at the vessel wall (Al-Khazraji et al., 2016). Under normal conditions in large arteries, blood flow has a parabolic velocity profile and the magnitude of shear stress ranges from 10-40 dynes/cm² (Traub et al., 1998).

The interplay between flow sensing and flow regulation is critical for maintaining normal vascular function. Moreover, the proper alignment of ECs is critical for proper EC function and regulation of vasomotor tone. The sensing of shear stress by the endothelium induces changes in EC morphology, function, and gene expression (Ando et al., 2009, Traub et al., 1998). In response to laminar shear stress, ECs become spindle-shaped and elongate their main axis parallel to the direction of blood flow (Tzima et al., 2001). At the genetic level, shear stress influences the expression of many genes through the activation of transcriptional factors (Ohura et al., 2003). Shear stress induces the expression of the gene transcription factor Krüppel-like factor 2 (KLF2), which mediates the cell alignment to flow and is essential for the formation of actin filament shear fibers (Boon et al., 2010).

In contrast, ECs lining vessels with stagnant or turbulent flow have a cobblestone-like appearance and lack a uniform orientation (Langille et al., 1981). This impairs EC function and leads to the development of vascular diseases including hypertension and atherosclerosis (Zhou et al., 2014).

Several studies have attempted to clarify the mechanisms by which the mechanical signals are transduced into the endothelium. However, this process remains not well understood and controversial (Ando et al., 2013). Multiple candidates have been suggested to sense shear stress including EC ion channels, receptor-tyrosine kinases including VEGFR2 and angiopoietin receptor (Tie-2), GTP binding protein-coupled receptors, adhesion molecules, glycocalyx, primary cilia, caveolae, and the cytoskeleton (Ando et al., 2013).

Furthermore, shear stress sensing is critical for the remodeling and optimization of vascular network architecture and lumen diameter. It is interesting that tyrosine kinase receptors can be phosphorylated by shear stress, since these receptors play major roles in
regulating hierarchical differentiation, EC specification, morphology, and migration (Davies, 1995).

1.3.3 Regulation of Capillary Perfusion and the Local Oxygen Content Control Loop

Dynamic regulation of terminal arteriole tone is fundamental for optimizing the delivery and distribution of O$_2$-carrying RBCs into capillaries (Bloch et al., 1982, Arpino et al., 2017). Fine-tuning of terminal arteriole tone is necessary to optimize O$_2$ transport directly at the site of oxygen delivery. The field’s understanding of perfusion at the capillary level has shifted from the regulation of RBC delivery in individual capillaries, to the regulation of RBC delivery throughout entire capillary networks.

A single terminal arteriole and its downstream capillary mesh constitute a microvascular unit (Bloch et al., 1982, Emerson, 1997 #906). The architecture of a microvascular unit dictates its ability to effectively regulate capillary perfusion. The fractal and hierarchical branching pattern of normal microcirculation is critical for ensuring the proper distribution of O$_2$-carrying RBCs into individual capillaries within a microvascular unit, and reduces plasma skimming at bifurcations (Ellis et al., 1994, Koller et al., 1987, Pries et al., 1989, Frame et al., 1993). In contrast, fractal branching patterns are not replicated in abnormal microvasculatures like the ones found in tumors, rendering a hypoxic tissue environment (Baish et al., 2000).

Heterogeneity in RBC delivery throughout capillaries supplied by the same feeder arteriole is necessary for tightly matching the delivery of O$_2$ to highly localized metabolic requirements (Duling et al., 1987, Ellis et al., 1994). This is particularly important in skeletal muscle, as individual muscle fibers within skeletal muscle have a variety of metabolic requirements at rest (Kusters et al., 2016). In the case of increased blood flow demand, a higher and more uniform RBC distribution is supplied to the capillaries. Once a terminal arteriole has maximally dilated, perfusion into its downstream capillaries is no longer controlled at the arteriole level. Instead, it is determined by passive rheological parameters of the RBC flow path including branching angles, flow path length, and flow resistance (Sarelius, 1986).
Importantly, the local and temporal regulation of capillary perfusion is intrinsically linked to local tissue O$_2$ content, independent of mechanical forces (Segal, 2005). Hypoxia is a powerful stimulus for enhancing delivery of RBCs in skeletal muscle (Jia et al., 2011, Parthasarathi et al., 1999). Regulatory mechanisms that rely on flow-mediated and hormonal responses do not directly sense O$_2$ levels and are thus alone insufficient to regulate the local supply of O$_2$ at the site of delivery (Ellis et al., 2012).

Interestingly, in addition to their role as the primary O$_2$ carriers in the blood, RBCs can regulate their own delivery and distribution (Ellis et al., 2012, Clifford, 2011, Ellsworth et al., 2012, Ellsworth et al., 2009). When RBCs enter a tissue region with high O$_2$ demand, O$_2$ will diffuse from the blood into the tissue and decrease the O$_2$ saturation of hemoglobin. This decrease in O$_2$ saturation stimulates the rapid release of ATP from the RBC from pannexin 1 channels in a linearly proportional manner (Ellsworth et al., 1995, Ellsworth, 2004). Since RBCs are in very close proximity to the endothelium in capillaries (<1 µm), the released ATP can diffuse across the short distance between the RBC and the endothelium, and interact with purinergic receptors on the surface of ECs (Ellis et al., 2012).

This interaction induces the release of NO and prostacyclin, as well as conducted Ca$^{2+}$ signaling and hyperpolarization via K$^+$ efflux in the endothelial cells. These vasodilatory signals are conducted upstream, resulting in increased blood flow and oxygen supply to the regions of increased O$_2$ demand (Ellis et al., 2012). Thus, the density of the capillary bed and surface area for RBCs to signal with the endothelium may play a crucial role in regulating the distribution of RBCs within microvascular units.

### 1.4 Blood Vessel Growth

#### 1.4.1 Developmental Vasculogenesis and Angiogenesis

In the embryo, de novo blood vessels form from primordial blood islands in a process called vasculogenesis (Flamme et al., 1997). The first blood islands arise from hemangioblasts between two mesoderm germ layers around E7-E7.5 in the mouse embryo (Patan, 2000). Blood islands harbor EC precursors called angioblasts, as well as hematopoietic precursors. Angioblasts accumulate and build de novo primitive tubes that
form the primitive vascular plexus (Figure 1.2). De novo tubes initially form as separate units that end up joining together. Moreover, angioblasts also migrate inside the embryo to form primitive vascular plexuses in distant organs.

The primitive vascular plexus is believed to expand into an extensive circulatory network by sprouting angiogenesis (Adams et al., 2007). Angiogenesis is defined as the process by which new blood vessels develop from a pre-existing vasculature. Over time this network remodels into a differentiated network of arteries, capillaries, and veins.

### 1.4.2 Sprouting Angiogenesis

Sprouting angiogenesis is the best-understood and most extensively studied process of neovascularization. The majority of the field’s knowledge regarding the processes and mechanisms regulating sprouting angiogenesis arise from studies in developing zebrafish embryos or mouse retinas. Sprouting is the dominant mode of neovascularization in these systems.

The main purpose of sprouting angiogenesis is to generate new microvessels and capillaries in tissue regions with insufficient blood flow. Vessel sprouts are EC-containing tubes that lack proper mural cell coverage until further maturation takes place (Limbourg et al., 2009). By generating new vessel sprouts, RBCs and O₂ can be delivered to non-vascularized areas.

For sprouting to occur, the activation of ECs from a basal quiescent state is required. This entails the breakdown of EC junctions, increase of vessel permeability, and the release of proteins contained in unique EC intracellular organelles called Weibel-Palad bodies (WPBs) (Rondaij et al., 2006). Proteins contained in WPBs play pro-angiogenic roles in ECs and are required for the rapid angiogenic response of ECs. Proteins are released from WPBs within seconds of exposure to secretagogues including VEGF, sphinogsine-1 phosphate (S1P), and thrombin (Matsushita et al., 2005, Matsushita et al., 2004). The specific proteins released from EC WPBs include vWF, P-selectin, fibrinolysis molecules, and Angiopoietin-2 (ANGPT2).
Figure 1.2 Vasculogenesis and sprouting angiogenesis

A. Vasculogenesis occurs in the embryo to generate de novo blood vessels. Hemangioblasts form blood islands that harbor endothelial and hematopoietic progenitors. Endothelial progenitors form primitive blood tubes that expand into an extensive primitive vascular plexus. B. Sprouting angiogenesis entails the migration of EC tip cells towards a gradient of angiogenic factors that are produced in hypoxic tissues. VEGF binds to and phosphorylates VEGFR2 on tip cell filopodia, stimulating the migration of the tip cell towards a chemical growth factor gradient. Tip cells signal to their neighbouring stalk cells through the Dll4-Notch pathway instructing them to proliferate and extend the length of the sprout, while also suppressing migratory behaviour.
A

Hemangioblasts → Blood Islands → Primitive Vascular Plexus

- Hemangioblasts
- Endothelial Precursors
- Hematopoietic Progenitors
- Endothelial Cells

B

Hypoxic Tissue

Angiogenic Factors

- VEGF
- pVEGFR2
- Blood Flow

Tip EC
Stalk EC
Phalanx or Non-Angiogenic EC
Pericyte
Sprouting angiogenesis entails a multistep branching morphogenesis program that generates new vessel channels (Caussinus et al., 2008). Sprouts extend out of existing vessels while maintaining contact with the parent vessel. The formation of a vessel sprout entails the specification of endothelial cells into three distinct and specialized phenotypes: tip cells, stalk cells, and phalanx cells (Gerhardt et al., 2003) (Figure 1.2). Tip cells guide the angiogenic sprout by migrating and becoming polarized in response to environmental cues and angiogenic growth factor gradients (Ribatti et al., 2012). The sprout tip contains numerous filopodial extensions that guide the direction of migration and are 100 nm – 100 µm in length. In contrast, stalk cells trail behind the tip and proliferate in order to extend the length of the vessel sprout. They also establish junctions with neighbouring ECs and generate basement membrane components. Phalanx cells represent the most quiescent ECs, and line vessels once new sprouts have reconnected to the vasculature. They generate tight junctions and align in a phalanx formation, reminiscent of the organization employed by ancient Greek soldiers (Mazzone et al., 2009).

Once sprouts have formed, they connect with other sprouts or existing vessels via vessel anastomosis. Several mechanisms have been suggested for the formation of a lumen in sprouting ECs. These mechanisms include intracellular vacuolization in a process termed cord hollowing, and the attainment of EC basal-apical polarity (Xu et al., 2011). However, the precise mechanisms dictating how a lumen forms in angiogenic sprouts remains debated.

1.4.3 Intussusceptive Angiogenesis

Although current understanding of new vessel formation stems primarily from powerful studies of sprouting angiogenesis, an alternate but underappreciated method for generating new blood vessels exists. This process is known as intussusceptive, or “splitting”, angiogenesis (IA).

IA was first described within the developing rat lung microvasculature in 1986 (Caduff et al., 1986). Caduff et al. observed no vessel sprouts in the developing microvasculature from postnatal day 1 up to day 270 when lung maturation is complete.
Instead, Caduff et al. observed small holes within the corrosion-casted alveolar microvasculature that were 1-2.5 μm in diameter. These small holes were first detected on day 4 during the formation and expansion of lung alveoli and remained frequently observed up to day 13, with many larger holes evident on day 270. Between birth and day 270, the developing rat lung capillary density increases by 35-fold (Caduff et al., 1986).

It was later discovered in the chick chorioallantoic membrane (CAM) angiogenesis model that these holes evident in casting were in fact tissue “pillars” spanning the lumen of microvessels (Patan et al., 1993). Transluminal tissue “pillars” are thus vascular structures <5 μm in diameter that are generated by a localized intraluminal protrusion of ECs which create a zone of contact with the opposite wall (Patan et al., 1993) (Figure 1.3).

For vessel growth to occur by IA, a single vessel is divided or “split” into two distinct and smaller vessels following the generation of transluminal EC pillars (Djonov et al., 2000) (Figure 1.3). This entails ECs making contact between opposite walls of a microvessel, perforation of the EC pillar core, and invasion of the core by perivascular cells and extracellular matrix, as demonstrated in both CAM and mouse tumor models (Patan et al., 1993, Djonov et al., 2000, Paku et al., 2011) (Figure 1.3). Pillars have also recently been identified in the abnormal vessels that form in skeletal muscle following local delivery of VEGF. In this case, there is enlargement of pre-existing microvessels and emergence of pillars that increase in size by extending along the long-axis of a vessel, and also fusing with neighbouring pillars (Gianni-Barrera et al., 2013). This divides and increases the number of vessels. The precise mechanism of pillar extension is unknown (Mentzer et al., 2014). Notably, EC pillars do not exhibit typical sprouting tip cell characteristics such as filopodia or migration towards a chemical gradient (Mentzer et al., 2014). Moreover, in contrast to sprouting, pillar formation does not disrupt the vessel basement membrane and is believed to be a non-proliferative process.
Intussusceptive angiogenesis entails the formation of translumenal EC pillars in response to the increased blood flow. Specifically, this pillar generation entails one or more ECs making a physical connection between opposite walls of a microvessel. Pillars then divide a single vessel and flow path into two or more daughter vessels of smaller caliber.
Increased Blood Flow

Daughter Vessels
The roles of pillar formation appear to be: 1) to induce microvascular growth by dividing an existing vessel, 2) to modify the architecture and arborization of microvascular networks by shifting the branching angle at bifurcations, and 3) microvascular pruning by eliminating redundant vessels or those with poor perfusion (Mentzer et al., 2014). Thus, although not well studied, IA appears to be an important method intrinsic to the microvasculature for optimizing blood flow.

In contrast to sprouting angiogenesis, the cellular and molecular mechanisms regulating IA are poorly understood. There are a limited number of studies investigating this type of angiogenesis. Currently, reported signals involved in generating EC pillars include activation of the endothelium by increased blood flow and shear stress, and vascular endothelial growth factor A (VEGF-A) (Djonov et al., 2002, Gianni-Barrera et al., 2013) (Figure 1.3). Importantly, pillars develop within a flowing blood stream and increased blood flow has been demonstrated to stimulate longitudinal capillary splitting (Egginton et al., 2001). However, using computational simulations based on the blood flow in CAM vasculature, pillars have also been predicted to develop primarily in micro-regions of vessels with low shear. In this case, pillar development is limited in surrounding regions of higher shear stress (Lee et al., 2010). This suggests that while increased shear stress can lead to the initiation of the IA process, there is an interplay between high and low shear stress as well as a permissive role of focalized low wall shear stress in pillar development (Lee et al., 2011).

To date, IA has been observed in the development of mammalian bone, retina, myocardium, kidney, ovary, and the CAM (Hirschberg et al., 2005, Burri et al., 2002, van Groningen et al., 1991, Makanya et al., 2005, Macchiarelli et al., 2006, Djonov et al., 2001, Djonov et al., 2000). In addition, it has been identified during physiologic neovascularization in response to increased shear stress during exercise within adult healthy skeletal muscle (Egginton et al., 2001). As well, IA has been identified during compensatory microvascular growth following injury to the rat lung and mouse retina (Konerding et al., 2010, Taylor et al., 2010), and during pathological neovascularization within tumors (Styp-Rekowska et al., 2011, Paku et al., 2011).
1.4.4 Arteriogenesis and Collateralization

Angiogenesis is necessary for delivering blood and O₂ to ischemic and regenerating tissues. However, this process alone has a limited capacity to increase blood flow and blood volume to the surrounding tissues (Limbourg et al., 2009). For this to occur, the process of arteriogenesis is required. Arteriogenesis entails the enlargement and remodeling of pre-existing collateral arteries into larger diameter conductance vessels for increasing blood flow, in addition to the arterialization of capillaries following ischemic injury (Schaper, 2009, Mac Gabhann et al., 2010) (Figure 1.4)

In contrast to angiogenesis, the remodeling response is not driven by hypoxia but by increased fluid shear stress caused by the occlusion of major arteries and diversion of flow into collateral vessels (Heil et al., 2004, Eitenmuller et al., 2006). It is characterized by the proliferation of ECs and SMCs, upregulation of genes for adhesive molecules and cytokines, and a complex process of lumenal expansion and vessel wall thickening to support an increase of blood flow and shear stress (Arras et al., 1998, Kondoh et al., 2004, Deindl et al., 2001, Pasyk et al., 1982). Both angiogenesis and arteriogenesis are important for adaptation and regeneration following ischemic injury.

1.4.5 Molecular Regulation of Angiogenesis

1.4.5.1 Initiation of Angiogenesis by Hypoxia

The most potent initiator of angiogenesis and neovascularization is tissue hypoxia (Carmeliet, 2000). Hypoxia’s actions are mediated through the gene transcription factor Hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric basic helix-loop-helix protein that induces the expression of pro-angiogenic molecules in hypoxic cells (Forsythe et al., 1996, Carmeliet et al., 1998). Its function is crucial for inducing tumour angiogenesis (Folkman, 2002). Furthermore, HIF-1 has been shown to play a role in ischemic skeletal muscle angiogenesis, mainly by modulating VEGF expression (Carmeliet, 2000, Pugh et al., 2003, Gustafsson et al., 1999, Milkiewicz et al., 2004, Ameln et al., 2005, Manalo et al., 2005, Nakano et al., 2007, Gavin et al., 2000).
Figure 1.4 Arteriogenesis and collateralization

Arteriogenesis entails the enlargement and remodeling of collateral arteries that receive little to no flow under normal conditions. Following occlusion of a major artery, such as the femoral artery that is indicated in the figure, blood flow is diverted into collateral arteries. In response to heightened flow, the collateral arteries expand their lumens and remodel to compensate for this. Shown are a normal mouse hindlimb (above), and a mouse hindlimb following femoral artery excision (below) that has been perfused with blue latex. The tissue was cleared in order to visualize the hindlimb arterial network filled with blue latex. These images demonstrate the enlargement of collateral vessels (arrowheads) following an increase in blood flow induced by excision of the femoral artery, denoted by x. Arrows indicate the direction of blood flow.
Shear Stress-Induced Remodeling

Deep Femoral Artery

Femoral Artery and Vein

Anterior Tibial Artery and Vein

Excised Femoral Artery and Vein

Collateral Vessels
The activity of HIF-1 is delicately controlled by local oxygen content (Brahimi-Horn et al., 2009). The active form of HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. Under normoxic conditions, HIF-1α is subjected to hydroxylation by prolylhydroxylase family members, which target the unit for ubiquitination and degradation (Maxwell et al., 1999). Under hypoxic conditions, HIF-1α is stabilized and dimerizes with HIF-1β, upon which it translocates to the nucleus and activates the transcription of over 60 genes, many of which are involved in the activation sprouting angiogenesis including VEGF-A (Forsythe et al., 1996).

1.4.5.2 VEGFR2 Signaling

1.4.5.2.1 VEGF-A - VEGFR2 Signaling

The vascular endothelial growth factor (VEGF) family consists of VEGF-A, -B, -C, -D and placental growth factor (PIGF) (Koch et al., 2011). VEGF-A was first described as a potent vessel permeability factor (Clauss et al., 1990). Later it was discovered that the binding of VEGF-A to vascular endothelial growth factor receptor 2 (VEGFR2) is the most powerful inducer of angiogenesis and is required for proper vascular morphogenesis (Carmeliet et al., 1996). Deletion of a single allele of VEGF-A leads to flawed vasculogenesis and angiogenesis, and is embryonic lethal (Carmeliet et al., 1996).

VEGFR2 is a membrane-bound tyrosine kinase receptor consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular kinase domain (Koch et al., 2011). Activation of VEGFR2 is critical for regulating EC survival, proliferation, migration, and vessel formation (Olsson et al., 2006). Activation of VEGFR2 via the binding of its ligand VEGF-A induces receptor homodimerization and leads to autophosphorylation of the critical tyrosine residues 1054 and 1059 in the intracellular kinase activation domain (Simons, 2012).

Autophosphorylation at these key residues is required for allowing maximal VEGFR2 kinase activity (Kendall et al., 1999, Dougher et al., 1999). Moreover, phosphorylated 1054 is a general marker of VEGFR2 kinase activation (Lanahan et al., 2010, Kendall et al., 1999). Phosphorylation of the residues 1054 and 1059 engages
various adaptor molecules required for the phosphorylation of other key residues in VEGFR2, such as tyrosines 1175 and 1214. This leads to the changes in EC phenotype and behaviour through the activation of downstream p38 MAPK, PI3K, and Rac signaling pathways (Koch et al., 2011).

The VEGF-VEGFR2 signaling axis is the master controller of sprouting angiogenesis, including the synchronization of EC sprouting activity (Kawamura et al., 2008). For vessel sprouts to form, ECs must increase vessel permeability, increase proliferation, and increase migratory behaviour. Importantly, the VEGF-A-VEGFR2 signaling axis regulates all of these processes (Olsson et al., 2006). Downstream of VEGFR2 activation the Src kinase and eNOS pathways modulate cell-cell contact and increase permeability (Olsson et al., 2006). EC proliferation is induced by the activation of Shc, PLCγ, PKC, and ERK pathways (Olsson et al., 2006, Pedram et al., 1998). EC migration is induced by p38 MAPK, HSP27, FAK, Paxillin, Rac, and IQGAP1 pathways (Koch et al., 2011, Yamaoka-Tojo et al., 2004, Olsson et al., 2006). The PI3K/Akt signaling pathways also upregulate anti-apoptotic genes that promote EC survival (Gerber et al., 1998).

EC tip cell selection within a pre-existing vessel is regulated by VEGF-A – VEGFR2 signaling (Gerhardt et al., 2003). The EC that locally expresses the highest level of VEGFR2 in the most sensitive to VEGF-A and is driven to become a tip cell (Gerhardt et al., 2003). Moreover, activation of Cdc42 GTPase by VEGF-A triggers the formation of filopodia in tip cells (De Smet et al., 2009). Filopodia help guide the elongation and directionality of the tip cell towards a chemical gradient of matrix-bound VEGF-A. In contrast, endothelial stalk cells only proliferate in response to VEGF-A activation for the purpose of elongating the sprout, but they do not migrate (Gerhardt et al., 2003). Interestingly, EC phenotypes during sprouting are amazingly plastic. ECs dynamically compete for the tip cell position during sprouting, where the differential expression of VEGFR2 and downstream Notch signaling over time induces a dynamic position shuffling of tip and stalk cells. This results in the regular exchange of the leading tip EC (Jakobsson et al., 2010).
VEGF-A - VEGFR2 signaling is also important for the induction of arterial-venous specification (Lawson et al., 2002). The VEGF pathway participates in AV specification by differentially regulating the activation of downstream targets in arterial and venous ECs, due to their differential expression of VEGFR2 and VEGFR3 and binding affinity for VEGF ligands (Lawson et al., 2002).

1.4.5.2.2 Ligand-Independent VEGFR2 Signaling

VEGFR2 can also be phosphorylated in a ligand-independent fashion. VEGFR2 exhibits rapid VEGF-independent phosphorylation in response to blood flow shear stress (Chen et al., 1999, Jin et al., 2003). For this to occur, the formation of an EC-specific mechanosensory complex is required (Tzima et al., 2005, Coon et al., 2015, Warren et al., 2014, Simons, 2012, Shay-Salit et al., 2002).

The EC mechanosensory complex is comprised of CD31/PECAM-1, VE-Cadherin, and VEGFR2. This mechanosensory complex is necessary for conferring proper flow shear stress responsiveness to ECs, the EC alignment to flow, and for EC survival (Tzima et al., 2005, Coon et al., 2015). These responses are necessary for the remodeling of the primitive vascular plexus in the embryo and for regulating vasomotor tone (Coon et al., 2015, Langille et al., 1986). CD31, which is enriched at EC-EC junctions, directly transmits mechanical blood flow force and is phosphorylated by Src-kinase in response to shear stress (Conway et al., 2012, Tzima et al., 2005). Once CD31 is phosphorylated, VE-Cadherin functions as an adaptor molecule and recruits VEGFR2 to the complex by binding directly to its transmembrane domain. Once recruited, VEGFR2 is phosphorylated by Src-kinase. VEGFR2 is phosphorylated at tyrosine 1054 within 15 seconds upon flow initiation in EC culture (Tzima et al., 2005). However, the specific details regarding how the mechanosensory complex is fully assembled are currently unknown.

In contrast to VEGF-induced VEGFR2 activation, ligand-independent phosphorylation of VEGFR2 increases vessel stability, decreases EC proliferation, and decreases EC migration. Following shear-induced VEGFR2 phosphorylation, the adaptor molecules Shc, Grb2 and Sos are recruited to the complex. This in turn activates
downstream PI3K, Akt, and p38 MAPK signaling pathways that increase cell-cell contact and decrease vessel permeability (Chen et al., 1999). Moreover, this converts integrins to a high-affinity state to mediate EC alignment and increases gene expression of KLF2 (Jin et al., 2003, Tzima et al., 2005). In addition, activation of Rac1 by shear stress in ECs mediates both cytoskeletal reorganization and affects gene expression (Tzima et al., 2002). Furthermore, knockdown of VEGFR2 expression induces a decrease of EC alignment to flow in vitro, even in the presence of VEGF, demonstrating the requirement for ligand-independent VEGFR2 phosphorylation for conferring flow responsiveness (Coon et al., 2015, van der Meer et al., 2010).

Whether ligand-independent VEGFR2 activation stimulates EC tip cell formation in sprouting angiogenesis is currently unknown. However, increased blood flow and shear stress are known stimulate IA in healthy skeletal muscle capillaries (Egginton et al., 2001). The role of ligand-independent VEGFR2 has not been fully elucidated in this process. However the activation of p38 MAPK, which is phosphorylated downstream of shear-induced VEGFR2 activation, has been suggested to be required for shear stress-induced IA in healthy mouse skeletal muscle in response systemic prazosin delivery (Gee et al., 2010).

1.4.5.3 Dll4 - Notch Signaling

Notch signaling is an evolutionarily conserved pathway that is involved in a wide range of biological processes including cell fate determination, proliferation, and cardiovascular development (Roca et al., 2007). Signaling through the Notch pathway occurs through direct cell-cell contact and communication. In mammals, there are four Notch receptors: Notch1-4. The Notch receptors have five ligands, three being a part of the Delta protein family (Dll1, Dll3, and Dll4) and two a part of the Jagged protein family (Jag1 and Jag2).

The Notch pathway regulates sprouting angiogenesis by controlling tip cell selection at the angiogenic front (Hellstrom et al., 2007, Suchting et al., 2007). Phosphorylation of VEGFR2 in tip cells induces the overexpression of Dll4. Dll4 is a transmembrane protein and Notch ligand. Dll4 binds to Notch1 on the surface of
neighbouring stalk cells. Notch1 signaling suppresses tip cell behaviour by reducing the expression of VEGFR2 and Dll4, thereby reducing the EC’s sensitivity to VEGF (Hellstrom et al., 2007, Holderfield et al., 2008, Jakobsson et al., 2010). In addition, VEGFR1 expression is also induced by Notch signaling, which reduces VEGF ligand bioavailability in stalk cells and limits tip cell formation and migration.

Jagged1 (Jag1) ligand antagonizes Dll4-Notch1 signaling and controls the number of endothelial sprouts and tip cells (Benedito et al., 2009). Jag1 is found in stalk cells at the sprout base, and is an proangiogenic regulator that antagonizes Dll4-Notch signaling and positively regulates sprouting by reducing endothelial tip cell Notch activation.

1.4.5.4 FGF - FGFR Signaling

Besides VEGF, the fibroblast growth factor (FGF) family is a potent promoter of embryonic and sprouting angiogenesis. FGF activation of FGFR signaling is critical for embryonic vascular development and precedes the appearance of VEGF signaling (Murakami et al., 2011). FGF2-induced endothelial tube formation is mediated by ERK1/2 (Kuzuya et al., 1999). Furthermore, FGF signaling is required for the maintenance of blood vessels in the adult (Murakami et al., 2008).

FGF signaling controls expression of VEGFR2 expression in endothelial cells by activating the VEGFR2 enhancer (Murakami et al., 2011). Basal stimulation of the endothelium by FGF is required for maintenance of VEGFR2 expression and the ability to respond to VEGF stimulation and accounts for the hierarchic control of vascular formation by FGFs and VEGF (Murakami et al., 2011).

In adult tissues, FGFs play important roles in angiogenesis and cancer. FGF2 promotes an angiogenic phenotype in endothelial cells that includes increased proliferation and migration (Presta et al., 2005). FGF2 has been shown to stimulate a powerful angiogenesis response within subcutaneous Matrigel implants (Frontini et al., 2011). As well, FGF2 stimulates mural cells to migrate and acts as a mitogen for ECs, SMCs, and fibroblasts (Gospodarowicz, 1974). Compared to VEGF-A, FGF2 induces blood vessels that are less leaky and more normalized.
1.4.6 Vessel Stabilization and Maturation

1.4.6.1 Mural Cells

Mural cells are essential for the stabilization and maturation of the thin neovessel wall formed during angiogenesis (Jain, 2003). Stabilization and maturation of the vessel wall involves the recruitment of pericytes and SMCs following the development of an endothelial tube. Mural cells deposit surrounding extracellular matrix and basement membrane to fortify structural stability, as well as elastic laminae in arterial vessels (Yin et al., 2015). Moreover, mural cell recruitment inhibits excessive angiogenesis and terminates the dependence of VEGF-stimulation for EC survival in newly formed blood vessels (Benjamin et al., 1998). As well, pericytes can abolish an angiogenic EC phenotype by inducing EC quiescence and inhibiting EC migration by paracrine and cell-cell contact-dependent control mechanisms (Orlidge et al., 1987).

In addition, mural cells are required and for increasing the complexity and hierarchical organization of vessels (Jain, 2003). Faulty or incomplete mural cell recruitment during embryonic development leads to inconsistent vessel diameters, EC hyperplasia, and diminished EC AV differentiation (Hellstrom et al., 2001). Overall, the recruitment of mural cells to newly formed microvessels is fundamental for proper vascular development and function.

1.4.6.2 PDGF – PDGFR Signaling

Platelet-derived growth factor (PDGF) is a fundamental mediator for the stabilization of nascent vessels. Pericytes and SMCs can be recruited to neovessels through several signaling pathways, and one of the best characterized is the PDGF-PDGFRβ signaling pathway. PDGFs are produced in active ECs and act as a mitogen and chemoattractant for cells of mesenchymal origin including pericytes, SMCs, and their progenitors (Shinbrot et al., 1994). PDGF-BB ligand binds to and activates the receptor PDGFRβ, which is highly expressed by pericytes and SMCs (Bjarnegard et al., 2004).

Signaling through the PDGF-BB – PDGFRβ axis has been demonstrated to be necessary for recruiting pericytes and SMCs to the developing vasculature in the embryo (Lutolf et al., 2005, Hellstrom et al., 1999). Moreover it is required for the wrapping of
angiogenic neo-vessels in tissue implants and the ischemic mouse hindlimb (Frontini et al., 2011). Inhibition of PDGFRβ signaling leads to embryonic lethality, as the lack of vessel stabilization results in dilated and thin-walled vessels that are leaky. Furthermore, a loss of this signaling in adult PDGF-BB and PDGFRβ knockout models impairs blood flow and increases tissue markers of hypoxia including VEGF (Hellstrom et al., 2001). An absence of pericytes leads to defects in endothelial tube maintenance and homeostasis, whereas a lack of wrapping SMCs renders vessels non-vasoresponsive.

1.4.6.3 Angiopoietin – Tie Receptor Signaling

The Angiopoietin family is critical for regulating vessel stabilization and maturation. Angiopoietins are a family of four secreted proteins that signal to Tie receptors (Sato et al., 1993). Tie receptors are expressed on the EC plasma membrane, whereas pericytes, SMCs, and fibroblasts express Angiopoietin-1 (ANGPT1) (Davis et al., 1996). ANGPT1 binds to and phosphorylates the Tie2 receptor. Loss of Ang1 or Tie2 is embryonic lethal and renders a poorly structured and destabilized vasculature (Sato et al., 1995). When ECs are in contact, Tie2 is located at the cell-cell interface and inhibits vessel permeability by preventing monolayer destabilization. Stimulation of EC Tie2 with ANGPT1 also induces the recruitment of SMCs to nascent channels in a PDGFRβ-independent signaling pathway (Uemura et al., 2002).

In addition to their role in vascular stabilization, angiopoietins also have pro-angiogenic roles by destabilizing the vasculature. Angiopoietin-2 (ANGPT2) destabilizes vessels by reducing pericyte coverage, and can lead to vessel regression (Huang et al., 2009). During active angiogenesis, ANGPT2 binding to the Tie2 receptor facilitates endothelial sprouting in the presence of VEGF. ANGPT2 is upregulated by local hypoxia and by proangiogenic growth factors including VEGF and FGF (Mandriota et al., 1998).

Importantly, PDGF-BB can simultaneously increase the expression of ANGPT1 and reduce the expression of ANGPT2 in mural cells through post-transcriptional destabilization, preventing ongoing or excessive vessel destabilization induced by ANGPT2 (Phelps et al., 2006). Clearly, the balance between ANGPT1-Tie2 and ANGPT2-Tie2 signaling is critical for determining the fate of a newly formed vessel.
1.4.6.4 Fibroblast Growth Factor 9

Fibroblast growth factor 9 (FGF9) is a mesenchyme-targeting growth factor that is necessary for embryonic development of the lung, heart, and cerebellum (Lavine et al., 2006). It was first identified in gliomas, and embryonic tissues have been found to exhibit a widespread expression of FGF9 (Miyamoto et al., 1993). In the embryo, the role of FGF9 has been investigated during coronary vessel development, where it signals to cardiomyoblasts to initiate a wave of signaling via the upregulation of sonic hedgehog (Shh) signaling, as well as the expression of VEGF and Angiopoietins. Together, these are required for proper coronary vessel growth (Lavine et al., 2006).

FGF9 is noteworthy in the context of vascular stabilization and maturation because unlike other FGF family members, it has no direct effect on endothelial tube formation. Instead, it promotes the stabilization of nascent vessel channels (Frontini et al., 2011). FGF9 promotes the ordered layering of pericytes and smooth muscles around new blood vessels in ischemic muscle through the activation of Shh signaling and the selective amplification of PDGFRβ-expressing mesenchymal cells (Frontini et al., 2011). This was associated with vasomotor competency and vessel longevity.

1.5 Model Systems for Studying Angiogenesis In Vivo

1.5.1 Zebrafish

The zebrafish is a classic model for studying vasculogenesis and sprouting angiogenesis during development. The zebrafish is also a powerful genetic tool for dissecting and understanding the molecular mechanisms regulating pathological angiogenesis (Ellertsdottir et al., 2010). It is also important for studying the processes of AV specification, lumen formation, and vascular regeneration. This because the vasculature rapidly develops within the zebrafish and in a highly reproducible manner. The dorsal aorta and vein form by vasculogenesis, and new sprouts called intersegmental vessels later develop from the dorsal and migrate towards the cardinal vein (Isogai et al., 2001).
The zebrafish tissue is optically transparent which enables easy visualization of blood vessels. Moreover, there is an abundance of transgenic lines that report the vasculature by fluorescent protein expression. This enables noninvasive visualization of the vasculature and allows for time-lapse imaging of growing vessels by brightfield or confocal microscopy. Moreover, the small size and abundance of zebrafish enables high throughput screening. The zebrafish can also be sectioned and immunostained to study the vasculature at cellular and molecular levels of resolution.

1.5.2 Chick Chorioallantoic Membrane

The avian embryo is another classical model for studying developmental vasculogenesis and angiogenesis in-vivo. The most widely used avian angiogenesis model is the chick chorioallantoic membrane (CAM). The CAM is a highly vascularized structure situated right below the eggshell (Baum et al., 2010). Careful opening of the eggshell creates a window to view the CAM. From days 3-10 after fertilization, ECs rapidly develop a vascularized network by sprouting, and then an angiogenic switch leads to growth and remodeling by intussusceptive angiogenesis (Ribatti et al., 2001). The ease of access, reproducible vascular pattern, and the ability to take repeated measurements over time makes the CAM suitable for the study of pro- and anti-angiogenic drugs.

Inspection of the CAM vasculature is classically performed by vascular corrosion casting for evaluating network architecture, in addition to intravital microscopy following intravascular perfusion with fluorescent dyes or particles for studying the formation of neovessels and blood flow dynamics over time (Lee et al., 2010). This makes the CAM model suitable for evaluating flow-dependent remodeling of the vasculature.

1.5.3 Retina

The ex-vivo model of angiogenesis in the developing mouse retina is one of the most widely used models for evaluating the molecular control of sprouting angiogenesis. Most of what is known about the cellular processes and molecular regulation of sprouting angiogenesis has been derived from studying the developing vasculature in the retina.
Angiogenesis in the mouse retina begins only after birth and in the central region of the retina at the optic nerve head. The microvascular architecture of the retina is unique in the sense that it is organized in a two-dimensional planar arrangement (Pitulescu et al., 2010, Hellstrom et al., 2007). The vasculature expands from the center of the retina towards the periphery by sprouting angiogenesis and tip cell formation. While sprouting continues at the angiogenic front, the vessels near the center remodel and form arteries and veins. Arteries and veins extend radially from the central optic nerve towards the retinal periphery at regular intervals. Arteries bifurcate laterally along their length into capillary plexuses that are highly branched and form a web-like meshwork (Eilken et al., 2010). The capillary plexuses are aligned precisely with horizontal neural and astrocytic laminae. Although sprouting is the predominant form of angiogenesis in the retina, IA can also occur as a vascular adaptation response to hypoxia in the adult (Taylor et al., 2010).

Key advantages of the ex-vivo retina model for studying angiogenesis are three-fold. First, all components of the vascular tree are organized within a flat two-dimensional network, allowing the simultaneous visualization of the all types of vessels within the network. Second, the direction of the angiogenic wave is highly reproducible and enables the easy identification of sprouting tip cells at the forefront of the vascular wave, and the simultaneous assessment of vessel remodeling near the center. The thin and optically transparent retina is an ideal tissue for visualizing vessels by three-dimensional confocal laser scanning microscopy after whole-mount immunostaining ECs and mural cells. Finally, it is a powerful system for studying vessel growth and remodeling in disease models, and following genetic knock-out or knock-in intervention.

1.5.4 Tumor

Tumors aggressively recruit a vascular network to sustain their growth (Kamoun et al., 2010). At first, growing tumors obtain their O₂ and nutrients from blood vessels that are within diffusion limits in the surrounding tissue. Over time, the tumor grows to a point where diffusion is insufficient for O₂ to the reach the core. At this point, the tumor becomes hypoxic and activates an angiogenic switch. This response to hypoxia results in
the massive infiltration of new vessels into the central regions of the tumor. However, the blood vessels formed in tumors are irregular and unorganized and are unable to effectively deliver O₂ (Yin et al., 2015). Perfusion in tumours is spatially and temporally heterogeneous, with slow RBC velocities and chaotic flow paths (Kamoun et al., 2010,Yin, 2015 #151).

Classically, studying tumor blood vessels is performed by histology and immunostaining strategies. Fluorescent antibodies targeted to EC markers, or intravascular perfusion with fluorescently conjugated lectins and subsequent microscopic imaging, are commonly used techniques for assessing tumor vessel density, organization, and diameter. For evaluating tumor perfusion, cranial or mammary fat pad widow chambers overlying subcutaneously implanted tumors allows for direct examination of in vivo tumor angiogenesis over time (Huang et al., 1999). With the incorporation of fluorescently labeled blood cells or microspheres, the tumor vasculature can be observed at high resolution using real-time epifluorescence, laser scanning, or multiphoton fluorescence microscopy (Kamoun et al., 2010). Moreover, these techniques can be used in combination with the delivery of pro- or anti-angiogenic compounds to test their efficacy on stimulating or abolishing tumor angiogenesis, respectively.

1.5.5 Bone

The bone microvasculature is formed exclusively by angiogenesis (Sivaraj et al., 2016). In the mouse, blood vessels invade the developing bone by sprouting angiogenesis on E13.5-E14.5, and vascular growth is completed in adolescence (Maes et al., 2010). The release of pro-angiogenic factors from chondrocytes located at both ends of a developing long bone guide growing vessels along the bone’s longitudinal axis. These vessels remodel into two distinct capillary beds with differential morphology and function (Sivaraj et al., 2016). Blood vessel formation and bone ossification are completed in tandem.

Bone, like skeletal muscle, is a highly vascularized tissue that contains a hierarchical microvascular network. Afferent arteries enter bone and branch into an extensive capillary network within the bone core, which then drain into a large vein in the
center of the bone marrow (Kusumbe et al., 2014). Interestingly, however, long bone contains two subtypes of capillaries that are regionally dispersed, albeit interconnected into a single meshwork with peculiar organization. In the bone metaphysis, capillaries exhibit a columnar and densely-packed parallel organization, and are hierarchically upstream from the bone marrow capillaries which form a highly branched and sinusoidal network (Sivaraj et al., 2016). Interestingly, arterioles directly connect only to the capillaries within the bone metaphysis and venules drain blood from capillaries within the bone marrow. This particular architecture renders two distinct metabolic environments within the bone, with well-oxygenated and hypoxic environments within the bone metaphysis and marrow, respectively.

The angiogenesis and vessel remodeling process during bone repair in the adult is not well understood but involves ingrowth of blood vessels from surrounding compact bone (Sivaraj et al., 2016). The hierarchically upstream metaphysis capillaries consist of a CD31-high endomucin-high EC population that becomes activated upon injury or hypoxia. These cells give rise to neovessels in bone (Kusumbe et al., 2014). Intravital imaging has revealed that neovascularization in murine bone involves the extension and anastomotic fusion of EC buds, which are functionally equivalent to endothelial sprouting tip cells seen in other organs, but exist in the form of rounded “buds” instead of tips with filopodia (Ramasamy et al., 2016).

1.5.6 Skeletal Muscle

Compared to angiogenesis during development, and the angiogenesis that occurs in the aforementioned organs and model systems, the angiogenesis process in adult skeletal muscle is not well understood. This is the case even though skeletal muscle is known to exhibit a robust neovascularization response in both physiologic and pathologic conditions (Hudlicka et al., 1992, Wagner, 2001). This is partly because tools for studying angiogenesis in-vivo in other organs are less suited for studying angiogenesis in skeletal muscle, and skeletal muscle tissue is not optically transparent.

The physiologic angiogenesis response in skeletal occur can occur in response to exercise and skeletal muscle contraction. Exercise induces perturbations in local oxygen
content and hemodynamics within the muscle. Intraluminal vessel stimulation by increases in blood flow shear stress following intraluminal administration of prazosin, and extraluminal stimulation by myocyte stretch and contraction, lead to an increase in skeletal muscle capillary density (Egginton et al., 2001, Zhou et al., 1998, Williams et al., 2006, Brown et al., 2003, Haas et al., 2000, Prior et al., 2004). Increased intraluminal shear in response to prazosin delivery induces capillary division by IA, whereas external mechanical stimulation of the endothelium leads to the breakdown of basement membrane and sprouting angiogenesis (Egginton et al., 2001). In these scenarios, pillars and tip cells have been identified by high-resolution electron microscopy. Skeletal muscle contraction also produces VEGF within myocytes and VEGF-A is required for exercise-induced angiogenesis (Olfert et al., 2010).

A common system for studying angiogenesis in ischemic skeletal muscle is in the mouse hindlimb following femoral artery ligation or excision (Limbourg et al., 2009). Moreover, the model mimics aspects of human PAD and thus is ideal for studying ischemia-driven neovascularization in muscle. The femoral artery is the major feeder artery for the mouse hindlimb. The femoral artery branches into the profunda femoris artery, which divides into collateral arterial vessels within muscles of the proximal hindlimb (Limbourg et al., 2009). The model uses this distinct anatomy to investigate arteriogenesis within the aforementioned collateral vessels as blood flow is diverted here following permanent blockage of the femoral artery. Furthermore, the model induces ischemia and angiogenesis in distal hindlimb muscles following the permanent blockage of blood flow, and is the site used for studying ischemia-induced neovascularization. The C57Bl/6 strain has the most robust arteriogenesis and angiogenesis response to femoral artery excision (Couffinhal et al., 1998, Scholz et al., 2002, Helisch et al., 2006, Duvall et al., 2004).

To assess the response to femoral artery excision laser Doppler imaging, whole mount pigment particle perfusion, histological strategies, and Doppler ultrasound are employed. Laser Doppler imaging of the paw has indicated that within 28 days following femoral artery excision bulk blood flow returns to near-normal levels via the vigorous opening and remodelling of collateral vessels (Helisch et al., 2006). In addition,
histological techniques have indicated that angiogenesis concomitantly occurs in infarcted hindlimb muscles distal to the surgical site, and the ischemic muscle has an end-stage microvascular density higher than that of healthy muscle (Limbourg et al., 2009).

Importantly however, very little is known about how new blood vessels form in skeletal muscle following ischemic injury. Studies using the hindlimb ischemia model have detected an increase in skeletal muscle capillary density by histology and immunostaining, but precisely how these new capillaries formed is not known. To date, only one study has shown direct evidence of sprouting tip cells within a partially infarcted ischemic muscle by whole-mount immunostaining (Al Haj Zen et al., 2010). Whether de novo vasculogenesis occurs in adult skeletal muscle remains controversial, as pro-angiogenic progenitor cells have been identified in skeletal muscle after ischemic injury but their precise role in promoting neovascularization is unclear (Aicher et al., 2007, Leroyer et al., 2009, Asahara et al., 1999, Ziegelhoeffer et al., 2004).

Furthermore, the literature is unclear as to whether angiogenesis occurs in mouse models of minor ischemia, in the absence of myocyte necrosis and subsequent myogenesis. Prolonged, moderate flow reduction to skeletal muscle does not necessarily lead to inflammation or extensive capillary growth, and the existing microcirculation remains intact (Brown et al., 2003).

Importantly, the currently widespread tools and strategies used to study angiogenesis and evaluate microvascular blood flow in ischemic muscle, such as laser Doppler imaging and histology, lack the spatial and temporal resolution necessary to directly visualize and measure neo-microcirculatory architecture, hierarchy, connectivity, RBC transit and distribution, vasoreactivity, and flow control. Therefore, the network-level architecture and physiology of newly formed microvessels in ischemic or necrotic and regenerating skeletal muscle is unknown. Thus it has not yet been explicitly determined whether the network architecture and function of a newly formed microvasculature is properly equipped to optimize blood flow at the site of oxygen delivery.
1.6 Therapeutic Angiogenesis

Treatments for patients with PAD and CLI include managing systemic atherosclerosis and surgical and catheter-based revascularization. Therapeutic angiogenesis is another approach that has been tested in clinical trials (Annex, 2013). The inherently strong capacity of skeletal muscle to regenerate (Carlson, 1973, Tedesco et al., 2010) with an associated angiogenesis response to ischemia (Couffinhal et al., 1998, Limbourg et al., 2009) has formed the rationale for therapeutic angiogenesis in patients with PAD. Therapeutic angiogenesis encompasses strategies that promote or augment the development of new microvessels as means to treat patients with ischemic vascular disease. The overarching goal of this therapy is to increase oxygen delivery to ischemic tissues that have been rendered hypoxic by compromised perfusion, and promote the restoration of normal tissue function. Another goal of therapeutic angiogenesis is to promote the regeneration of injured tissues (Madeddu, 2005). Regenerative angiogenesis is critical for restoring perfusion to damaged tissues that are attempting to regenerate following ischemic injury and necrosis.

Research in these areas has focused on returning bulk blood flow to affected organs via surgical intervention and angiogenic growth factor delivery methods. Growth factor delivery has demonstrated promising results in animal models by potently stimulating neovessel growth, leading to their testing in clinical trials. For critical limb ischemia these growth factors include plasmid or adenoviral injection of VEGF-A, FGF1, FGF2, FGF4, and HGF (Annex, 2013, Tongers et al., 2008).

Despite their promise, there has been little success in translating these preclinical results into benefit for patients with peripheral or coronary vascular disease to date (Simons et al., 2002, Henry et al., 2003, Norgren et al., 2007). These trials have shown indications of short-term benefit in limb function, however the benefits do not last. Patient selection and dosing concentration may entail some of the reasons for the lack of therapeutic success.
1.7 Anti-Angiogenic and Vascular Normalization Therapy

Tumors recruit a vascular network to sustain their growth. This response underlies the rationale for the use of anti-angiogenic agents for cancer therapy (Chung et al., 2010). In 1971, Judah Folkman coined the term anti-angiogenic therapy, which entails promoting the regression or destruction of tumor vessels so that they cannot sustain their growth (Folkman, 1971). However, despite an appealing premise and some clinical success, anti-angiogenic therapy has not transformed cancer management. As monotherapy, and combined therapy using multiple tyrosine kinase inhibitors, these anti-angiogenic drugs have only been beneficial for few types of tumors and have not improved patient survival (Ebos et al., 2011).

The less than anticipated advances with anti-angiogenesis approaches suggest that the relationship between tumor vessels and tumor behavior is more complex than originally considered (Jain, 2005). Important in this regard is that as described in Section 1.2.3.2., tumor vessels do not resemble normal vessels. This limits the delivery of chemotherapeutic drugs to the tumour cells and blunts the response to radiation therapy (Jain, 2005). With these limitations in mind, an alternative approach to targeting the tumor vasculature has been proposed, based not on obliterating tumor vessels but on normalizing them. Several studies have demonstrated that tumor vessels have the capacity to improve their integrity following anti-VEGF therapy (Tong et al., 2004). Furthermore, normalization therapy induces the improvement of mural cell wrapping and reduction of vessel density (Goel et al., 2011). These findings suggest that normalizing instead of obliterating the tumor vasculature may improve cancer therapy by preventing metastasis and increasing the delivery of chemotherapy drugs.

1.8 Tools for Studying Microvascular Perfusion

Numerous tools are currently available for evaluating microvascular perfusion, and each has it’s own advantages and limitations. Measurements include qualitative and quantitative indices of bulk flow, flow velocity, and flow distribution. The focus of this
section will be on tools typically employed for evaluating microvascular perfusion in the mouse hindlimb, with a brief overview of existing tools used in the clinic.

Laser Doppler imaging is an optical approach for investigating perfusion in mouse models of hindlimb ischemia. The approach directs laser light into the tissue. The light is scattered by moving RBCs and produces a frequency shift produced by the Doppler effect, and is detected by the scanner to measure velocity (Briers, 2001). Laser Doppler imaging is capable of evaluating changes in superficial perfusion over time following femoral artery excision, as it is sensitive only to perfusion in the upper 200-300 µm of the skin (Jakobsson et al., 1993). Therefore it is unable to evaluate perfusion within deeper tissues, and is limited by low spatial and temporal resolution. Because of this, it is not capable of measuring blood flow in individual microvessels, or perfusion at the capillary level. Instead, it provides only an average indication of the magnitude and distribution of flow within the hindlimb. It also cannot discriminate the local return of flow to an arteriogenesis or angiogenesis response. An advantage of this technique however is that it is non-invasive and can take repeated measurements of the same animal over time.

Doppler ultrasound is a non-invasive method used for studying angiogenesis and measuring tissue perfusion. Doppler ultrasound entails the acoustic detection of moving particles within the blood stream (Leong-Poi et al., 2003). Intravascular microbubble delivery used in conjunction with Doppler ultrasound imaging allows quantitative measurement of microcirculatory blood flow and pulsatility (Leong-Poi et al., 2003). Moreover, they can be targeted to surface EC markers to localize signals to active areas of vessel growth. This tool has been used to evaluate the magnitude of perfusion in hindlimb ischemia mouse models. However, like laser Doppler imaging, it suffers from low spatial resolution at cannot directly measure flow at the site of oxygen delivery.

MR imaging has been used to study changes in tissue perfusion and permeability over time that are associated with angiogenesis. Regional tissue perfusion can be assessed with MR imaging after administration of gadolinium-based contrast agents or arterial
spin labeling (Luo et al., 2002). However, this approach lacks sensitivity for targeted molecular imaging and has a limited ability to directly measure microcirculatory flow.

Optical coherence tomography (OCT) is a high-resolution and three-dimensional technique for detecting blood flow in tissues. OCT entails illuminating tissue with light of low coherence and detecting back-scattered light based on coherence matching between the incident and reflected beams (Ntziachristos, 2010). This strategy has been used to map regional perfusion and vascular architecture up to 3 mm deep in hindlimb ischemia models (Jia et al., 2011, Poole et al., 2013). However, this technique lacks sensitivity for targeted molecular imaging and is unable to image microvessels that are less than ~20 µm in diameter (Mariampillai et al., 2012).

Importantly, these described techniques cannot evaluate perfusion directly at the capillary level. In contrast, intravital microscopy does have the power to study capillary architecture and perfusion in living tissues. Intravital microscopy is an invasive technique that can provide powerful cellular, anatomic, and functional insight into vascular physiology and disease (Jain et al., 2002, Fukumura et al., 2008, McDonald et al., 2003, Taqueti et al., 2013, Vajkoczy et al., 2000, Lehr et al., 1993, Menger et al., 1993). There are many approaches to intravital microscopy including brightfield transillumination, fluorescence epi-illumination, multiphoton microscopy, and spinning disk confocal imaging. Each has its own application to different tissues and disease processes.

An overarching tenet of my thesis is that intravital video microscopy has the potential to study angiogenesis at the resolution necessary for advancing the understanding of skeletal muscle angiogenesis. Furthermore, it has the potential to discover the processes and targets required for successful postnatal angiogenesis, and serve as a platform for testing strategies for augmenting microvascular function. This is because intravital microscopy can image individual microvessels at high spatial and temporal resolution. Therefore, intravital microscopy has the potential to afford an unprecedented window into capillary-level architecture and hemodynamics.

Intravital microscopy has been developed for studying human blood flow and malignant disease, but only recently. This entailed developing a novel intra-operative
microscopy platform with a robotic arm to inspect the tumor microcirculation at the capillary level, including the use of fluorescent dyes to generate contrast for visualizing RBCs (Fisher et al., 2016). As well, intravital fluorescence lifetime imaging has been used in humans since 1999 for studying penetration of nanoparticles and skin cell metabolism (Leite-Silva et al., 2013), and endoscopic autofluorescence strategies for distinguishing normal and cancerous tissues. Other tools that are used in the clinic for evaluating perfusion, but are typically uncommon, include nail-fold sidestream darkfield imaging and capillaroscopy (Goedhart et al., 2007), sublingual orthogonal polarization spectral imaging (OPS) (Martin et al., 2009), laser Doppler flowmetry (Debbabi et al., 2010), and Doppler optical microangiography (Baran et al., 2015). Development of minimally invasive intravital microscopy could hold exciting potential.

1.9 Aims of the Thesis

The foregoing review highlights the considerable body of research in the field of vascular regeneration. The underlying premise of my thesis is that, despite the current state of the art, an understanding of the architecture and function of the micro-neovasculature at both the network level and functional level remains poor.

To address this research gap, the broad goals of my thesis were to: 1) develop approaches and strategies to interrogate the newly formed microvasculature in adult mice, at high spatial and temporal resolution, including real-time; and 2) to uncover the architectural and functional attributes of these networks, attributes that define the extent to which they can appropriately delivery RBCs to the tissues.

I present my data in three Chapters:

**Chapter 2 Overview:** My objective was to determine the microvascular network architecture, hierarchy, and RBC transit dynamics in a model of orthotopic mouse renal cell carcinoma. Using intravital microscopy and a novel real-time strategy to elucidate the vasoreactivity, I uniquely ascertained the microcirculatory dynamics in this tissue and the extent to which it could be improved by the growth factor, FGF9.
Chapter 3 Overview: My next objective was to determine the physiological attributes of the regenerated microcirculation in the ischemic mouse hindlimb. For this, I undertook femoral artery excision in the mouse and delineated the temporal profile of microvascular network architecture, hierarchy, and RBC transit in the regenerated extensor digitorum longus (EDL) muscle. In addition, I determined whether the regenerated microcirculation could regulate blood flow in response to local hypoxia and the SMC wrapping profile of the regenerated terminal arterioles.

Chapter 4 Overview: My objective in this chapter was to determine the micro-architectural events by which the earliest flowing neovessels in ischemic skeletal muscle convert into an arborized network. I undertook this by scrutinizing a critical early-stage neovascularization process with real-time RBC analysis, processed RBC transit mapping, high-resolution tissue histology, 3-dimensional reconstructions, and VEGFR blocking studies in vivo.

Collectively, my findings provide entirely new data and perspectives on the formation, vulnerabilities, and cellular dynamics of the newly forming microvasculature in tumors and ischemic and regenerating skeletal muscle.

1.10 References


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CHAPTER 2

Assessment of Microvascular Architecture and Function in Mouse Renal Cell Carcinoma Via Novel Real-Time Microscopy Strategy


2.1 Introduction

A highly organized and functional microvascular network architecture is fundamental for maintaining organ function and homeostasis. This includes a hierarchical arteriole-capillary-venule (ACV) network, single file transit of RBCs in capillaries, and local regulation of RBC transit into the network (Pries et al., 1995, Pries et al., 1996, Koller et al., 1987, Ellis et al., 1994, Segal, 2005).

Tools and approaches for assessing the microvasculature in tissues are limited. Currently used strategies such as laser Doppler imaging or tissue histology provide little information on microvascular network-level architecture or hierarchy, and not at high spatial resolution. Furthermore, these tools provide little to no information on microvascular perfusion, RBC transit dynamics, and vasoreactivity. Understanding of the architecture and function of microvascular networks under pathological conditions is particularly limited.

A primary goal of my thesis was to develop approaches to understand the microcirculation in pathological tissues. Tumors represent a pathological tissue with profoundly abnormal microvessels. As well, the tumor vasculature has gained major attention because local blood flow can determine tumor behaviour and malignancy (Rummelt et al., 1994, Goel et al., 2011). Because of this, tumor vessels are a target for therapy with the goal of killing tumor cells by obliterating tumor vessels (Folkman, 1971, Chung et al., 2010).
The fact that tumor vessels are also very abnormal provides an additional consideration into understanding tumor biology. The tumor microvasculature is typically characterized by dilated vessels and haphazard vessel branching which impairs O$_2$ delivery (Jain, 1988, Less et al., 1991, Nagy et al., 2009, Fukumura et al., 2010, Tong et al., 2004). In light of this, an alternative approach to targeting the tumor vasculature has been proposed. This approach is not based on the classical tactic of obliterating tumor vessels but, instead, on potentially normalizing the vessels and improving their capacity for enhancing perfusion (Tong et al., 2004, Mazzone et al., 2009). The goal of this normalization would be to kill tumor cells by improving the delivery of chemotherapeutic drugs and the tumor’s response to radiation treatment (Jain, 2005, De Bock et al., 2011).

In this chapter, I describe the development of a strategy for evaluating microvascular network architecture and blood flow, at high spatial and temporal resolution, in renal tumors in mice. This strategy entailed real-time imaging of RBCs in transit, using intravital video microscopy and following intravascular injection with high molecular weight fluorescently-labeled dextran. I also developed an in vivo approach to interrogate the vasomotor competence of tumor microvessels. Finally, I capitalized on a recent discovery that delivery of fibroblast growth factor 9 (FGF9) can productively modify skeletal muscle microvasculature (Frontini et al., 2011). I tested whether FGF9 could normalize tumor microvascular architecture, hierarchy, or vasoreactivity.

2.2 Materials and Methods

2.2.1 Cell Culture

Renca Cells (ATCC, CRL-2947), a transformed kidney cell line from BALB/cCR (Charles River) mice, were maintained in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum. Cultures of Renca cells expressing either GFP or human FGF9 were generated by infection with adenovirus expressing the respective cDNA for 16 h at 37°C.


2.2.2 Orthotopic Renal Carcinoma Model

Female BALB/c mice (Charles River) at 2 months of age were used and all experiments were conducted in accordance with the University of Western Ontario Animal Use Subcommittee. Implantation of Renca cells into the subcapsular space of female BALB/c mice yields an orthotopic tumor model that mimics the behavior of adult human renal cell carcinoma, including spontaneous metastases to the lung (Salup et al., 1985). Orthotopic primary tumors were generated from $2.5 \times 10^5$ Renca cells resuspended in phosphate-buffered-saline (PBS) and mixed 1:1 (v/v) with growth factor-reduced Matrigel (BD Biosciences). Cell-Matrigel suspensions were injected as 30-µL aliquots into the subcapsular space of the left kidney of BALB/c mice. On designated days, animals were euthanized with isofluorane inhalation and both kidneys harvested for histologic analyses. Renca cells were transduced with adenovirus encoding either GFP or human FGF9.

2.2.3 Intravital Microscopy

Tumor-bearing BALB/c mice were anesthetized with inhaled isoflurane, the kidney tumor was exteriorized and placed within the central recess of a custom-built transparent plexiglass chamber positioned on the stage of an inverted microscope (IX81, Olympus) and kept moist by filling the chamber with 0.5 mL of 37°C Krebs physiological salt solution, pH 7.4. The tumor was epi-illuminated with a 120 W Mercury X-Cite high-pressure bulb light source with 10x and 20x objectives (Olympus UPlanSApo). Blood flow in the tumor was visualized by either ultraviolet epi-illumination (DAPI U-MWU2: 330-385 nm excitation filter, 420 nm emission filter) or blue light epi-illumination (U-MWIBA2: 460-490 nm excitation filter, 510-550 nm emission filter) following intravenous tail vein administration of FITC-labeled dextran ($2 \times 10^6$ MW; 20 mg/mL, 50 µL, Sigma). Images were acquired using a cooled charge-coupled device camera (Rolera-XR, QImaging) at a rate of 21 images/s. Live image sequences were digitized and stored as uncompressed AVI movie files using custom frame capture software (Neo-Vision) and in-house software written in the MATLAB (Mathworks) programming environment. Offline analysis of movie files was conducted
using ImageJ (NIH) software to quantify the vascular network morphometry of at least 10 random fields of view, in both control and FGF9-exposed tumors. Vascular length density was determined by manual tracing of all vessel centerlines and total network length was expressed relative to tumor area. Branch point density was determined by manual point counting of all vascular bifurcations and expressed as total bifurcations relative to total network length. Lumen diameter was determined by manually quantifying widths of individual vessels, perpendicular to the vessel centerline. Capillaries were defined as vessels with single-file red blood cell flow. Microcirculatory units were defined as flow routes in which RBCs flowed from an arteriole-like vessel into one or more capillaries that drained into a venous structure characterized by slower flow than the feeding arteriole. Simple microcirculatory units were defined as those with a single capillary between an arteriole-venule pair and complex circulatory units were those with two or more capillaries between the pair. Vasoreactivity was assessed in arteries 20-60 µm in diameter, assessing the entire exposed tumor surface, at baseline and following administration of phenylephrine (Sigma, 1x10^-6 M), after flushing with Krebs solution and administering potassium chloride (KCl, 1x10^-2 M), and after flushing with Krebs solution and administering sodium nitroprusside (SNP) (1x10^-7 M).

2.2.4 Local Delivery of Anti-PDGFRβ Antibody to Renal Tumors

Seven days after Renca cell implantation, mice were anesthetized with isoflurane and the left kidney and associated tumor were re-exposed. Rat monoclonal PDGFRβ blocking antibody (16-1402-82, eBioscience) or rat IgG isotype-matched control (16-4231-82, eBioscience) were diluted to 50 µg/mL in 30% Pluronic F-127 gel (Sigma) and the gel was then applied to the tumor surface with two sequential applications, 30 s apart, using a fine brush. After 3 days the tumors were subjected to intravital microscopy, as above.

2.2.5 Statistics

Statistical analyses were performed using Prism 5 (GraphPad software). Values are presented as mean ± standard error of the mean (sem). Groups were compared by two-tailed t-test. Comparison of vessel diameters, which were not normally distributed,
was undertaken using Mann-Whitney testing. Frequencies of capillaries positioned between arterioles and venules, and arteries displaying vasoreactivity were compared using Fisher’s exact test.

2.3 Results

2.3.1 Generation of a Vascularized Tumor For Real-Time Microscopy Assessment

Colleagues in Dr. Pickering’s laboratory had previously demonstrated that implantation of the renal adenocarcinoma cell line, Renca, into the kidney subcapsular space of female BALB/c mice generates an orthotopic kidney tumor (Frontini, 2010) (Figure 2.1). Renca-derived tumors after 14 days were 3-fold heavier (514.9±114.6 mg) than contralateral control kidneys (146.8±10.2 mg). Microvessels were readily detectable in 14-day-old tumors, based on hematoxylin and eosin staining, and immunostaining revealed that over 90% of these microvessels were lined by CD31-positive endothelial cells (Frontini, 2010).

2.3.2 The Microvascular Architecture in Renal Tumors is Profoundly Disorganized

To evaluate and characterize the microvascular network architecture of Renca-derived tumors, I developed a real-time microvessel visualization approach. This entailed exteriorizing the kidney and surrounding tumor and imaging RBCs in transit by epifluorescence intravital microscopy (Figure 2.2 a). This revealed highly disorganized and chaotic RBC flow within the Renca tumor (Figure 2.2 b). RBC transit was strikingly slow, with RBCs tumbling through enlarged and tortuous vascular channels (Figure 2.2 b). In addition, the movement of RBCs throughout the microvascular network was seemingly random, characterized by frequent directional changes at vessel bifurcations and by opposing flow directions within two parallel vessels.

To make a broad comparison of the tumor microcirculation to that of a normal tissue, I also used a similar approach to study the microvascular network architecture in
Figure 2.1 Generation of a mouse orthotopic renal carcinoma

Photograph of a kidney tumor harvested 14 days after renal subcapsular injection of Renca cells in growth factor-reduced Matrigel. The corresponding, non-injected contralateral kidney is shown on the right. From: (Frontini, 2010).
Renal Carcinoma

Healthy Kidney
Figure 2.2 Network architecture of normal and abnormal microcirculations

A. Schematic of kidney tumor exteriorization and epifluorescence intravital video microscopy setup.

B. Still frame from an ultraviolet light epifluorescence microscopy video sequence showing the abnormal microvascular network architecture of the Renca-derived tumor.

C. Still frame from an ultraviolet light epifluorescence microscopy video sequence showing the structured hierarchy of arterioles (A), capillaries (C), and venules (V) in healthy mouse extensor digitorum longus (EDL) muscle.

D. Table of comparisons between network length density (µm vessel / µm² surface area), network branching density (number of bifurcations / mm vessel length), and vessel segment lumen diameter (µm) in Renca tumor and mouse EDL skeletal muscle. For length and branching density, 10 fields of view were averaged from three tumors and five hindlimb skeletal muscles. For lumen diameter, 346 microvessels from three tumors and 164 microvessels from five skeletal muscles were measured.
**A**

Surgical Exteriorization of Kidney and Tumor

Plexiglass Holding Chamber

Syringe-Accessible Chamber for Local Administration of Vasoactive Agents

Abdominal Wall

Kidney

Tumor

IV Tail Vein Injection of 2,000 kDa FITC-Dextran

**B**

Renca Tumor

**C**

Skeletal Muscle

**D**

<table>
<thead>
<tr>
<th></th>
<th>Vessel Length ($\mu$m/µm$^2$)</th>
<th>Branch Points (/mm vessel)</th>
<th>Lumen Diameter (µm)</th>
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<tbody>
<tr>
<td>Renca Tumor</td>
<td>0.032 ± 0.01</td>
<td>11.5 ± 1.1</td>
<td>31.8 ± 1.9</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.029 ± 0.01</td>
<td>4.3 ± 1.1</td>
<td>5.6 ± 0.1</td>
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(Normal Network)
the mouse extensor digitorum longus (EDL) muscle. This revealed that within the EDL muscle, RBCs flowed rapidly through highly organized, narrow-caliber vascular channels (Figure 2.2 c). Moreover, the movement of RBCs in neighbouring vessels occurred in a generally unified flow direction and was characterized by the flow of RBCs from arterioles to capillaries to venules (Figure 2.2 c). Therefore, the microvascular network of Renca-derived tumors contrasted strikingly with that of a healthy microcirculation in a normal tissue.

Quantitative assessment of microvascular network architecture revealed that the renal tumor and EDL muscle were both highly vascularized tissues (vascular length density: 0.032±0.01 vs 0.029±0.01 µm/µm², P=0.212) (Figure 2.2 b-d). However, the tumor network exhibited a high level of vessel branching (11.5±1.1 vs 4.3±1.1 bifurcations/ mm vessel, P<0.0001) (Figure 2.2 b-d). As well, the individual vessel segments were markedly dilated (31.8±1.9 vs 5.6±0.1 µm, P<0.0001) (Figure 2.2 b-d). Thus, both qualitatively and quantitatively, the microvascular network in Renca-derived tumors was profoundly abnormal.

2.3.3 Detection of FGF9-Mediated Changes to the Renal Tumor Microvascular Architecture

Delivery of FGF9 has previously been shown to productively modify the skeletal muscle microvasculature (Frontini et al., 2011). Therefore, I next questioned whether my imaging strategy could provide insights as to whether delivery of FGF9 could improve the tumor microvascular network architecture, recognizing the profoundly disorganized network I identified. To do this, Renca cells expressing either GFP or human FGF9 were implanted into the kidney subcapsular space of female BALB/c mice. Both GFP- and FGF9-expressing Renca cells yielded rapidly expanding local tumors. Colleagues in Dr. Pickering’s laboratory previously found that FGF9 had little to no direct effect on the growth of tumor cells (Frontini, 2010). Also, immunoblotting revealed that FGF9 expression was detectable in Renca-FGF9 tumors for at least 14 days, but FGF9 was not detected in control (GFP) tumor (Frontini, 2010). Furthermore, both tumor types were of equal weight (Frontini, 2010).
Imaging RBC transit in the tumors by intravital microscopy revealed striking changes to the microvascular network architecture imparted by local delivery of FGF9. Whereas vessels in control tumors were densely packed and highly branched, those in FGF9-expressing tumors were more spaced and evenly distributed with an overall less chaotic appearance (Figure 2.3 a,b). Quantitative analysis revealed a 33% reduction in microvessel length density (P=0.034), a 67% reduction in mean lumen diameter (P<0.0001), and 57% fewer branch points (P=0.019) (Figure 2.3 c).

As well, in contrast to the rough appearance of control tumor microvessels and the associated sluggish flow, there was a “smooth” appearance to some vessels in FGF9-expressing tumors with faster RBC transit. The latter suggested the emergence of an arterialized vessel (Figure 2.3 b). Consistent with this, there was heterogeneity in vessel architecture throughout the network, with both fast and slow flowing vessels. In the latter case, there were RBC-free spaces in the lumen, consistent with a venous structure (Figure 2.3 b). Therefore, delivery of FGF9 led to reduced vascularity, less branching, narrower channels, and the suggestion of more differentiated vessel types.

2.3.4 FGF9 Normalizes Microvascular Network Hierarchy and RBC Distribution

I next sought to determine the microvascular network hierarchy and physiologic vessel specification in the renal tumor microcirculation. To do this, I assessed blood flow routes throughout the tumor microvasculature by tracking the motion of RBCs using real-time microscopy at double magnification, which allowed for a more detailed assessment of individual RBC movement. In control GFP-expressing tumors, RBC transit was characterized by serpentine flow routes (Figure 2.4 a). As well, RBC movement from channel to channel was seemingly random (Figure 2.4 a). Moreover, transitions in vessel caliber in the direction of blood flow were non-progressive (Figure 2.4 a,b). That is, the transitions did not follow a large-smaller-larger network hierarchy pattern typical of a normal microcirculation with an ordered arteriole-capillary-venule hierarchy. Furthermore, there were substantial step changes in lumen diameter from parent to daughter vessel (Figure 2.4 b).
Figure 2.3 Productive modification of the tumor microvascular architecture by delivery of FGF9

A-B. Ultraviolet fluorescence intravital microscopy images of RBCs flowing within the vasculature of GFP-expressing (A) and FGF9-expressing (B) renal tumors 14 days after injection of Renca cells. GFP-expressing tumors (A) show a densely packed network of highly branched vessels. The network in FGF9-expressing tumors (B) is less dense and less branched, with the appearance of arterialized (A) and venous (V) structures.

C. Graphs depicting vascular length density (*p=0.034), lumen diameter (**) p<0.0001), and branch point density († p=0.019). At least 10 fields of view were averaged for each mouse, with 3 GFP-expressing tumors and 4 FGF9-expressing tumors.
GFP-Renca Tumor

FGF9-Renca Tumor

A

B

C

Vessel Length Density (µm/µm²)

Lumen Diameter (µm)

Branch Point Density (/ mm vessel)

GFP

FGF9

C

0

0.01

0.02

0.03

0.04

0

10

20

30

40

0

5

10

15

0.01

0.02

0.03

0.04

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10

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30

40

0

10

20

30

40

0

5

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15

0

10

20

30

40

0

10

20

30

40
Figure 2.4 FGF9 imparts hierarchy to the tumor vasculature

A. High magnification intravital image illustrating serpentine and chaotic flow routes (yellow arrows) in GFP-expressing tumors.

B. High magnification intravital image illustrating orphaned capillaries (white arrows) and lumen diameter step changes (dotted yellow lines) in GFP-expressing tumors. Yellow arrow depicts flow route.

C. High magnification intravital images of two distinct FGF9-expressing tumors with ordered flow through hierarchical arterial (A)- capillary (C)- venous (V) microcirculatory units. Arrows depict flow routes, and dotted arrows shows flow routes through arteriole located deeper within tissue.

D. Graphs depicting the density of simple and complex microcirculatory units of arterial to capillary to venous flow (*p<0.0001; † p=0.007), capillary density defined as vessels with single file RBC flow (p=0.233), and percentage of capillaries positioned within a microcirculatory unit (*p=0.028). At least 10 fields of view were averaged for each mouse, with 3 GFP-expressing tumors and 4 FGF9-expressing tumors.
In contrast to control tumors, I identified discrete hierarchical ACV microcirculatory units in FGF9-expressing tumors. In these units, RBCs flowed through gradually and progressively smaller caliber vessels, entered one or more capillaries, and then drained into a collecting venule (Figure 2.4 c). I termed the microcirculatory units that entered a single capillary as simple microcirculatory units and those entering more than one capillary as complex microcirculatory units.

Notably, within capillaries, RBCs transited in single file, with plasma gaps between each cell (Figure 2.4 b,c). By this definition, capillaries were rare in control tumors (Figure 2.4 d). Moreover, those that were identified were effectively orphaned, with no evidence for a feeder arteriole or a draining venule. Instead, they were bizarrely situated between two large caliber venous-like channels (Figure 2.4 b). In contrast, 30% of capillaries in FGF9-expressing tumors were appropriately positioned within a bona fide microcirculatory unit (P=0.028, Figure 2.4 d). Thus, delivery of FGF9 imparted hierarchy to the tumor microvasculature.

2.3.5 FGF9 Generates a Vasoreactive Tumor Vasculature

I next sought to determine whether the FGF9-modified microvascular tree was capable of regulating blood flow. In a normal microvascular network, this is accomplished through the vasoreactivity of arteriolar vessels. However, this has never been assessed before in a tumor microvascular network. To do this, I established a system for the local delivery of vasoconstrictive and vasodilatory agents to the exposed tumor surface (Figure 2.2 a). Ten days after implantation of GFP- or FGF9-expressing tumor cells, mice received a systemic injection of FITC-conjugated high molecular weight dextran, vessels were imaged by intravital microscopy, and phenylephrine (1x10^-5 M), potassium chloride (KCl, 1x10^-2 M), and sodium nitroprusside (1x10^-7 M) were sequentially administered. By recording video frames of the entire exposed tumor surface, I ascertained that although vasoresponsiveness was not evident in the control tumors, it was present at sites in FGF9-expressing tumors. In some vessels, discrete sites of vasoconstriction developed in response to phenylephrine (Figure 2.5 a). Constriction further increased upon KCl delivery, with more diffuse lumen narrowing (Figure 2.5 a).
Figure 2.5 FGF9 imparts tumor vessels with vasoreactivity

A. Intravital microscopy images of vessels within orthotopic renal tumors 10 days after injection of Renca cells, imaged live by intravital microscopy following injection of FITC-labeled dextran. Images depict the vascular lumen before and after subfusion of phenylephrine (10^{-5} M), KCl (10^{-2} M), and SNP (10^{-7} M), for a minimum of 3 min as indicated. Arrows within the lumen depict the direction of flow. Top panel, vessel within an FGF9-expressing tumor showing focal vasoconstriction (arrows) in response to phenylephrine subfusion, and both focal and diffuse constriction following KCl subfusion. The lumen diameter widens after subfusion with SNP. Middle panel, vessels within an FGF9-expressing tumor showing flow down a network with progressively smaller branches. Following phenylephrine subfusion, diffuse vessel constriction can be seen as well as complete cessation of flow in some of the distal vessel branches (short arrows). This is accompanied by a loss of fluorescence signal throughout the tumor, indicating widespread reduction in tumor perfusion. Diffuse vessel constriction, cessation of flow in distal vessels (short arrow), and generalized hypoperfusion are even more pronounced following KCl administration. These changes are partially reversed following delivery of SNP. Bottom panel, vessels within a GFP-expressing tumor showing no change in luminal diameter or flow indicators following delivery of vasomotor agents.

B. Plot of all vessel segments imaged, 20-60 \mu m in diameter and with continuous flow into progressively smaller branches, which displayed vasoreactivity. n=34 and 28 vessels in GFP- and FGF9-expressing tumors, respectively (p=0.0085).
A

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<th>Baseline</th>
<th>Phenylephrine $10^{-5}$ M</th>
<th>KCl $10^{-2}$ M</th>
<th>SNP $10^{-7}$ M</th>
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<tr>
<td><strong>FGF9</strong></td>
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<td><strong>GFP</strong></td>
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B

![Bar graph showing the number of feeder vessels in GFP-Tumor and FGF9-Tumor conditions, with non-vasoreactive and vasoreactive categories.](image)

- Non-Vasoreactive
- Vasoreactive

Number of Feeder Vessels

- GFP-Tumor
- FGF9-Tumor
After SNP, these changes were almost completely reversed and the vessels relaxed (Figure 2.5 a). In regions where an arborized and tapered arterial tree was present, vasoconstriction was sometimes associated with complete cessation of flow in one or more of the arterial branches. This was coupled to a corresponding diffuse loss of FITC signal in the surrounding tumor environment, suggesting reduced perfusion to the region (Figure 2.5 a, middle).

Quantitative analysis of all vessels between 20 and 60 µm in diameter that flowed into narrower caliber vessels confirmed a statistically significant acquisition of vasoreactivity in FGF9-expressing tumors (Figure 2.5 b). The ability to regulate flow through vasomotion, with functional α-adrenergic and nitric oxide signaling, represents a level of physiologic maturity not previously identified in tumor microvessels.

2.3.6 FGF9-Mediated Network Differentiation is Abrogated by Local Inhibition of PDGFRβ

I next applied the real-time microscopy strategy to help dissect the mechanism behind FGF9-driven microvascular network differentiation. Dr. Pickering’s laboratory had previously shown that delivery of FGF9 increases the expression of PDGFRβ (Frontini, 2010). Furthermore, using the Renca-derived tumor model, it was found that FGF9 selectively amplified a PDGFRβ-positive stromal mesenchymal cell population in the tumor, which were found to wrap endothelial cell-lined channels (Yin et al., 2015). Given the existence of an FGF9-sensitive, PDGFRβ-expressing stromal population, I asked if the PDGFRβ receptor mediated the FGF9-induced reshaping of the microvasculature. To test this, I developed a strategy to apply Pluronic-F127 gel containing anti-PDGFRβ antibody, or isotype-matched IgG, to the surface of 7-day-old tumor. I then assessed the impact of PDGFRβ blockade on network architecture using the intravital microscopy-based assessment of the vascular network three days later.

Exposure of FGF9-exposed tumors to PDGFRβ blocking antibody yielded vessels that were significantly more dilated (2.0-fold, P<0.0001) (Figure 2.6 a,b). Moreover, the tumor microvessels were more branched (1.7-fold, P=0.003) (Figure 2.6 a,b). Furthermore, the number of capillaries in FGF9-exposed tumors was reduced by 57%
Figure 2.6 PDGFRβ blockade abrogates the effects of FGF9 on tumor network architecture and vasoreactivity

A. Intravital microscopy image of FGF9-exposed tumors locally treated with control IgG or anti-PDGFRβ antibody, showing that PDGFRβ blockade (right) renders a disordered microvasculature that resembles a GFP-expressing tumor and contrasts the ordered arrangement of vessels in FGF9-exposed tumors (left). Specifically, a hyper-dilation of vessels (lumens constrained by yellow dotted lines), increased branching, and a lack of ordered network hierarchy are shown.

B. Graphs showing data from FGF9-incubated tumors exposed to anti-PDGFRβ antibody or control IgG, depicting vascular lumen diameter (*p<0.001), branch point density (*p=0.003), length density (p=0.094) or capillary density (*p=0.046). Data are averaged from at least 10 fields of view for each antibody treatment condition.
A

FGF9-Renca + IgG

FGF9-Renca + Anti-PDGFβ Antibody

B

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<tr>
<th>Lumen Diameter (µm)</th>
<th>IgG</th>
<th>Anti-PDGFβ</th>
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<th>Branch Point Density (in vessel)</th>
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<th>Vessel Length Density (µm/µm²)</th>
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<th>Capillaries (mm²)</th>
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(P=0.046) (Figure 2.6 a). In addition, by dynamically imaging vessel lumen caliber I found no evidence for constriction of microvessels in response to phenylephrine or KCl in FGF9-exposed tumors subjected to PDGFRβ blockade. Thus, the structural and functional actions of FGF9 on the tumor vasculature depended on PDGFRβ-positive stromal cells.

2.4 Discussion

In this study, I have demonstrated a powerful new approach for evaluating the renal tumor microvasculature in mice. Using epifluorescence intravital microscopy, and by tracking the movement of RBCs, this real-time strategy revealed profound abnormalities in RBC transit and network architecture, including an absence of microcirculatory hierarchy. Thus, the construction of an organized microcirculation for properly delivering RBCs into capillaries was not replicated during generation of the tumor microvasculature.

This microvascular imaging strategy for assessing tumor vasculature has several strengths. First, the approach enables rapid visualization of microvascular networks over a large area of tumor. Each field of view can be rapidly imaged (~15 seconds), followed by simply moving to the next field of view. Second, the strategy enables high spatial (0.6-1.3 µm² pixel size) and high temporal resolution (21 images/second) imaging. This provides a flow-based assessment of microvascular network architecture. Third, individual RBCs in transit can be delineated in real-time to assess network hierarchy, RBC transit dynamics, and vessel specification. Fourth, this strategy can be used in conjunction with the local delivery of vasoactive agents to assess microvessel vasoreactivity and changes in network perfusion, in real-time and in space. A limitation of this imaging strategy is that the microvasculature cannot be visualized within tissue that is deeper than ~50 µm from the tumor surface.

Real-time micro imaging of blood flow not only gave detailed insight into the deranged tumor vasculature, it also powerfully revealed the consequences of delivering FGF9 to the tumor. The paradigm of tumor vessel normalization has emerged in recent
years and it is proposed that anti-angiogenesis therapies may confer their benefit, at least in part, through vessel normalization (Goel et al., 2011). To date, indicators of vessel normalization have included reduced vessel density, smaller caliber channels, enhanced endothelial cell integrity, and increased coverage by mural cells (Goel et al., 2011, Kim et al., 1993, Yuan et al., 1996, Greenberg et al., 2008, Carmeliet et al., 2011). However, if the goal is to approximate the perfusion attributes of healthy tissue, there remains a substantial “gap” between the architectural and flow status of a post-anti-angiogenesis targeted vasculature and that of healthy tissues. The findings herein with FGF9 delivery indicate that this differentiation gap can be narrowed and that a physiologically advanced microcirculation in tumors is achievable.

The emergence of a hierarchical network following delivery of FGF9 where fast flowing arterioles fed capillaries that drain into slow flowing venules was particularly striking. A degree of vascular network hierarchy has been suggested in glioblastomas treated with cedranib, as indicated by a mixture of high- and low-flow vessels (Emblem et al., 2013). Hierarchy after anti-angiogenesis therapy in mice has also been suggested by improvements in tumor metabolism and drug delivery (Maes et al., 2014, Chauhan et al., 2012). However, these changes in blood flow and drug delivery could also be due to alterations in hydrostatic pressure (Goel et al., 2011). The current intravital, micron-level assessment of the vasculature provides direct evidence that microcirculatory hierarchy can be obtained in tumors. FGF9 yielded a network with reduced vessel density, reduced vessel diameter, reduced vessel branching, and hierarchical arteriole-capillary-venule microcirculatory units. This hierarchical architecture provides a functional route for flowing RBCs, avoiding recycling of deoxygenated blood.

Real-time visualization of all flowing RBCs enabled us to define capillaries as vessels in which RBC transit proceeded exclusively in single file. The emergence of bona fide capillaries has not been identified in tumors before. A seminal report by Tong and co-workers (Tong et al., 2004) described a reduction in vessel caliber in response to VEGFR2 blockade, with the emergence of more vessels below 20 μm in diameter. However, vessels in this size range can still accommodate simultaneous transit of multiple cells, unlike capillaries in normal tissues where diameters average 4-6 μm
We determined that capillaries with single file red blood cell flow were rare in control tumors and most of those that were found were orphaned from arterioles. By contrast, FGF9 generated bona fide capillaries appropriately situated within microcirculatory units to optimize oxygen delivery.

My finding of vasomotor competence in the microcirculation of FGF9-exposed tumors is also relevant to generating functional perfusion in tumors. Several integrated control systems regulate microvascular flow, including centrally initiated neural impulses, circulating hormonal factors, local myogenic responses, and retrograde conducted vascular signaling from local hypoxic cues (Segal, 2005, Clifford, 2011). The fact that arterial-caliber vessels in FGF9-exposed tumors contracted to α-adrenergic stimulus and dilated in response to nitroprusside, with concomitant shifts in distal perfusion, reveals a previously unobserved competency in regulating tumor microcirculatory flow. Although we cannot exclude the possibility that some of the vasoactive arteries were pre-existing vessels that became engulfed by the growing tumor, the widespread emergence of vasoreactivity exclusively in FGF9-exposed tumors argues for maturation of de novo generated tumor blood vessels.

The real-time intravital microscopy approach also established that the PDGFRβ was central to the productive vascular remodeling conferred by FGF9. Blockade of PDGFRβ in the tumors prevented the productive vascular remodeling by FGF9, and led to hyperdilation of the channels and reduction of bona-fide capillaries. As well, it abolished the vasoreactivity imparted to feeder vessels in FGF9-exposed tumors.

In summary, using a novel real-time microvascular imaging strategy, I have undertaken a flow-based characterization of tumor microvascular architecture, hierarchy, capillary flow, and vasoreactivity. This has revealed both profound abnormalities, but also the capacity to regain microvascular differentiation through the delivery of FGF9.

2.5 References


CHAPTER 3

Four-Dimensional Microvascular Analysis Reveals That Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation


3.1 Introduction

It is well established that a robust angiogenesis response can occur following ischemic injury to skeletal muscle. This neovascularization response, together with opening of collateral vessels, can restore blood flow to the otherwise compromised muscle (Couffinhal et al., 1998, Limbourg et al., 2009). The innate capacity to regenerate a vasculature in muscle has also provided an important rationale for efforts to augment angiogenesis for individuals with peripheral vascular disease. However, to date such strategies have met with little to no success (Annex, 2013, Simons et al., 2003). There may be several reasons for this lack of success, but a fundamental and unanswered question is the extent to which a regenerated microvasculature in adult muscle can meet the metabolic needs of the tissue.

The microcirculation in normal skeletal muscle consists of a highly-organized network of arterioles, capillaries, and venules, structurally arranged to optimize oxygen transport. This microcirculatory network provides the necessary conduits for red blood cells (RBCs) to deliver their oxygen. In addition, the network dynamically controls the passage of RBCs throughout the tissue. This local control system ensures that RBC delivery is tightly coupled to the metabolic needs of the tissue. An important driver of this control loop is the local oxygen content, which can inform the feeder arterioles to dilate or constrict as appropriate (Segal, 2005). Thus, integration of precise structural and functional attributes of the microcirculation are critical to oxygenating muscle tissues. However, the attributes of a regenerated microcirculation in skeletal muscle are largely
uncharacterized. The network microarchitecture is poorly understood and whether the network can dynamically regulate RBC transit is entirely unknown.

One of the best-studied models of regenerative angiogenesis following ischemia is ligation or excision of the femoral artery in mice (Limbourg et al., 2009). Use of laser Doppler imaging has established near complete return of bulk blood flow to the ischemic mouse hindlimb, particularly in the C57Bl/6 strain (Scholz et al., 2002, Helisch et al., 2006). This return of flow has been shown to be due to both collateral vessel opening (Couffinhal et al., 1998, Scholz et al., 2002, Helisch et al., 2006, Duvall et al., 2004) and generation of new microvessels (Limbourg et al., 2009, Scholz et al., 2002), as established from angiographic and histologic studies. However, standard histology techniques do not address network-level architecture of the microvasculature. As well, in vivo imaging tools typically lack the spatial resolution to resolve distinct microvessels or to measure blood flow within them. Accordingly, answering whether the regenerated microcirculation is optimized for oxygen delivery requires different methodologies that can provide higher spatial and temporal resolution and are suitable for evaluating skeletal muscle angiogenesis.

Herein, I describe an integrated, high-resolution 4-dimensional imaging approach for studying the regenerated microvasculature in skeletal muscle. I utilized this approach to determine if the reconstructed microcirculation recapitulates the anatomy and physiology of a normal microvasculature. I report that despite extensive angiogenesis and remodeling, the regenerated microcirculation is a profoundly flawed network that cannot adequately control the delivery of RBCs.

3.2 Materials and Methods

3.2.1 Mouse Hindlimb Ischemia

Experiments were conducted in accordance with the University of Western Ontario’s Animal Care and Use Subcommittee. Male C57BL/6J mice (Jackson
Laboratories, Bar Harbor, ME) 12 weeks of age were anesthetized with ketamine (80mg/kg) and xylazine (10mg/kg) administered intraperitoneally. Hindlimb ischemia was induced by ligating the right femoral artery above and below the profunda femoris branch using 6-0 silk sutures and excising the intervening 5-6 mm portion of artery (Limbourg et al., 2009, Frontini et al., 2011).

3.2.2 Histology and Immunostaining

Extensor digitorum longus (EDL) muscles were dissected, fixed in either Tris-buffered zinc or 4% paraformaldehyde (PFA) overnight and embedded in paraffin. Five-µm thick cross-sections of EDL muscle were stained with hematoxylin and eosin. Near adjacent sections were subjected to heat-mediated antigen retrieval with citrate buffer and immunostained for endothelial cells using biotinylated rat monoclonal anti-mouse CD31 antibody (1:100, BD Biosciences 553371) and DAB Substrate (Vector). Nuclei were visualized by counterstaining with hematoxylin. For immunofluorescence detection of endothelial cells, five-µm thick sections of EDL muscle were subjected to antigen retrieval and immunostained using rat monoclonal anti-mouse CD31 antibody (1:20, Dianova, Clone SZ31) or rat monoclonal anti-mouse endomucin antibody (1:100, Santa Cruz, Clone V.7C7, sc-65495) and detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:100 for CD31, 1:200 for endomucin, Life Technologies). Endothelial cells were also stained using biotinylated isoelectin-b4 (1:100, Sigma, BSI-B4, L2140), detected using Dylight 488-conjugated streptavidin (1:200, Vector) and immunostained for Von Willebrand factor (vWF). In the latter instance, EDL muscles were fixed in 4% PFA at room temperature for two hours, subjected to cryoprotection by immersion in 15% sucrose at room temperature for two hours then 30% sucrose at 4°C overnight, frozen in OCT compound (Tissue-Tek), and stored at -80°C. Ten-µm thick frozen sections were post-fixed with 4% PFA for ten minutes and immunostained using rabbit polyclonal anti-vWF antibody (1:50 in 0.1% Triton-x 100, Millipore AB7356) and biotinylated goat anti-rabbit IgG (1:100 in 0.1% Triton-X 100, Vector) and Dylight 488-conjugated streptavidin (1:200 in 0.1% Triton-X 100, Vector). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech, 0100-020). Sections were imaged by widefield microscopy (Olympus BX-51) and photomicrographs captured with Northern
Eclipse (EMPIX Imaging Inc.) software.

Capillary density and capillary-to-muscle fiber ratio were quantified in 10 equally spaced high-powered (60x objective) fields from a 50-µm thick zone at the muscle periphery using ImageJ (NIH). Muscle fiber density and cross-sectional area were quantified in the mid-zone of the native and regenerated EDL muscle under high-powered view (40x objective) using ImageJ (NIH). For infarct analysis of all muscles in entire the hindlimb, the PFA-fixed hindlimb was decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days and then divided into five blocks that were embedded in paraffin, sectioned, and stained for hematoxylin and eosin.

### 3.2.3 Detection of Muscle Hypoxia

Muscle hypoxia was assessed by immunohistochemical detection of pimonidazole adducts, following intraperitoneal injection of pimonidazole hydrochloride (60 mg/kg, Hypoxyprobe™-1Kit, Hypoxyprobe, Inc.) into mice 28 days following femoral artery excision. Forty minutes after injection, mice were sacrificed by isoflurane overdose, perfused sequentially with PBS and 4% PFA via left ventricle cannulation at physiological pressure, and both injured and contralateral EDL muscles were dissected. Five-µm thick paraffin-embedded cross-sections were immunostained with a monoclonal mouse anti-pimonidazole antibody (Hypoxyprobe-1 mAb, 1:50) and the signal detected according to the manufacturer’s protocol with DAB substrate (Vector). Injured EDL muscles dissected from mice 28 days following femoral artery excision that were not injected with pimonidazole hydrochloride served as a technical control. Sections were counterstained with hematoxylin and hypoxia signal was quantified from entire EDL muscle mid-zone cross-sections using ImageJ, by isolating DAB via the colour deconvolution plugin (Ruifrok et al., 2001) and measuring the raw integrated signal density following thresholding, using an identical grey scale (61-255) for all samples.

### 3.2.4 Transcript Analysis by RT-PCR

Total RNA from uninjured and regenerated EDL muscles 28 days following femoral artery excision was extracted with Trizol (Life Technologies) and RNEasy
(QIAGEN) and subjected to reverse transcription as described previously (Frontini et al., 2011). The mRNA abundance of adrenomedullin, lysyl oxidase (LOXL2), procollagen lysyl hydroxylase 2 (PLOD2), and VEGF-A was quantified using quantitative RT-PCR and SYBR Green chemistry and a ViiA 7 Real Time PCR System (Life Technologies). Gene expression was normalized to the mouse 18S signal using the ΔΔCt method. Primers pairs (OriGene) employed were: Adrenomedullin, 5’-GCCAGATACTCCTCGCAGTTC-3’ and 5’-GCGACTACTCTGCTGTCAAGGA-3’; LOXL2, 5’-TTCTGCGGAGGACACTGAGT-3’ and 5’-CGGTCACCTATCTGTAGTGCT-3’; PLOD2, 5’-CATCCGAGAGTTCATTGCCTCAG-3’ and 5’-CATGAGTGG ACTTTCTGTCGCG-3’; VEGF-A 5’-CTGCTGTAAACGATGAAGCCCTG-3’ and 5’-CCTCTCTACTCGAAGGATGTCG-3’; ANGPT1 5’-AACCGAGCCCTACTCAGTGCCG-3’ and 5’-GCATCCTCTCGTGCTGAAATCGG-3’; and ANGPT2 5’-AACCTCGCTCCTCAGGAAGCAGC-3’ and 5’-TTCCGCACAGTCTCAGAAGGTG-3’.

3.2.5 Intravital Video Microscopy

RBC transit in individual microvessels within a 50 µm-deep zone across the entire mouse EDL surface was assessed by epifluorescent intravital video microscopy (IVVM). Briefly, mice were anesthetized with ketamine and xylazine intraperitoneally and a longitudinal incision was made over the anterior hindlimb. Tibialis anterior and peroneus longus muscles were separated from surrounding fascia and spread apart to reveal the underlying EDL (Tyml et al., 1991). The EDL was covered with an 8x8 mm glass coverslip, and positioned face-down on the stage of an inverted microscope (Olympus IX81), maintaining body temperature at 37°C. After a 20-minute stabilization period, the EDL was epi-illuminated with a 120 W Mercury X-Cite high-pressure bulb light source, via a 10x objective (Olympus UPlanSApo). RBC transit was visualized by either ultraviolet light epi-illumination (DAPI U-MWU2: 330-385 nm excitation filter, 420 nm emission filter) or blue light epi-illumination (U-MWIBA2: 460-490 nm excitation filter, 510-550 nm emission filter) (Frontini et al., 2011, Yin et al., 2015) following intrapenile injection of FITC-labeled dextran (2x10^6 MW; 20 mg/mL, 30 µL, Sigma). Video
recordings (696x520 pixels, 21 images/s) were captured using a cooled charge-coupled device camera (Rolera-XR, QImaging) and displayed in real-time on a computer monitor for at least 315 images (15 seconds). For all studies, the entire EDL surface was recorded and analyzed (7-10 fields of view). Video sequences were digitized and stored as uncompressed AVI files for post-processing using custom acquisition software (NeoVision) and in-house software written in the MATLAB (Mathworks) programming environment.

RBC hemoglobin O\(_2\) saturation in capillaries near the EDL surface was assessed by white light transillumination IVVM, as described previously (Ellis et al., 2010, Fraser et al., 2012). For this, a silk suture was attached to the distal EDL tendon. The tendon was severed distal to the ligature, and the EDL was reflected onto the microscope stage (Olympus IX81) and secured at approximately its in situ resting length and orientation. The EDL was kept moist with Plasma-lyte 148 (Baxter International) at 37°C, and covered with polyvinylidene chloride film (Saran Wrap, Dow Chemical) and a glass coverslip to isolate it from room air. The EDL was transilluminated with a 100-W xenon lamp, and transmitted light was captured with 10x and 20x objectives (Olympus UPlanSApo) and a parfocal beam splitter (DualCam) fitted with 442-nm (an O\(_2\) sensitive wavelength for oxy- and deoxyhemoglobin) (Faber et al., 2003) and 454-nm (an isosbestic or O\(_2\) insensitive wavelength) band pass filters for dual video cameras. The beam splitter passed 442-nm light to one cooled charge-coupled device camera (Rolera-XR, QImaging) and 454-nm light to a second camera, and dual video recordings (696x520 pixels, 21 images/s) were simultaneously obtained using custom capture software (Neovision). Images from both cameras were registered using the beam splitter and the capture software ensured synchronized frame-by-frame acquisition from both cameras. A minimum of 20 fields of view (10 at the arteriolar end, and 10 and the venular end of a capillary network) were randomly selected and recorded for 60 seconds in each EDL muscle. Video sequences were stored as uncompressed PNG files for post-processing using in-house software written in the MATLAB (Mathworks) programming environment. O\(_2\) saturation in capillaries was obtained by analysis of PNG files using custom image analysis software written in Matlab, as described by Ellis et al (Ellis et al.,
1990, Ellis et al., 1992) and Japee et al (Japee et al., 2004, Japee et al., 2005). Briefly, in-focus capillary segments were selected, and the location of the vessel centerline was used to extract light intensity values from every video frame and generate space-time images for both the 442- and 454-nm wavelengths. The light intensity values were analyzed frame-by-frame to identify the location of each RBC and plasma gap within the capillary. The mean optical density (OD) of each RBC at both wavelengths, was computed as OD=log(I_o/I_m) were I_o is the measured light intensity of the plasma gaps (intensity of incident light) and I_m is the measured light intensity values for RBCs. The mean RBC hemoglobin O_2 saturation was determined from the ratio of OD(442)/OD(454), which is linearly related to O_2 saturation, i.e. SO2 = a + b * ODratio. The slope and intercept were determined from an in vivo calibration.

3.2.6 Analysis of Microvascular Network Architecture

Length density and bifurcation density of EDL muscle microvascular networks were quantified from RBC transit maps, which effectively displayed a micro-angiogram of all flowing microvessels, using ImageJ. RBC transit maps were generated from IVVM video sequences using custom MATLAB software as described (Japee et al., 2004, Varghese et al., 2005). From the videos generated by ultraviolet light epi-illumination, RBC transit maps were generated from “minimum” (MIN) images, which displayed the minimum light intensity value and therefore RBC transit at a given pixel during the duration of the video sequence. From the videos generated by blue light epi-illumination, RBC transit maps were generated from “sum of all differences” (SAD) images, which displayed the cumulative sum of the square of differences in light intensity values at each pixel between consecutive video frames, thus generating a single map of all microvessels perfused with RBCs and plasma. Length density (microvasculature length [µm] / EDL area [µm^2]) was quantified at each time-point via manual tracing of all vessel centerlines and normalizing total network length to EDL area. Bifurcation density was determined by manual point counting of all vessel bifurcations and normalizing to total network length. Arteriole-capillary-venule microcirculatory units and AV malformations were identified from blue light epi-illumination videos and corresponding SAD images.
3.2.7 Analysis of Capillary Network Hemodynamic Resistance

To assess possible changes in capillary network resistance, three control and three regenerated networks (all at day 28) were reconstructed using 10x IVVM data from fields of view acquired to determine RBC flow and network architecture, as described above. Each network was reconstructed into a collection of nodes and connecting cylindrical vessel segments using our previously developed software (Fraser et al., 2012). Two-phase (RBCs and plasma) steady-state blood flow simulations were performed using an established computational model (Pries et al., 1990, Pries et al., 1994, Goldman et al., 2000, Al-Khazraji et al., 2015) with a fixed pressure difference (DP) between all inflow and outflow nodes. The resistance for each network was calculated as DP divided by the total flow (Q_{tot}) through (i.e., into or out of) the network, \( R = \frac{DP}{Q_{tot}} \). Flow was mainly unidirectional (e.g., in at bottom and out at top of network), but for two networks flow was bidirectional indicating a major source (arteriole) or sink (venule) within the network. For these networks, resistances were calculated for upward and downward flowing portions separately and added to approximate the resistance of a single network with unidirectional flow. For all networks resistance was normalized by dividing by length (L) in the main flow directional and multiplying by width (W) in the perpendicular direction, so that \( R_{norm} = \frac{W \times R}{L} \). This was done to account for variability in network lengths and widths produced by the capture and reconstruction procedure, since resistance is proportional to vessel length and total flow should be proportional to width of a network.

3.2.8 Analysis of Capillary Red Blood Cell Velocity and Distribution

Red blood cell velocities (\( V_{RBC} \left[ \mu m/sec \right] \)) in individual capillaries exhibiting characteristic single-file RBC transit were quantified from video files using space-time images (Ellis et al., 1992, Japee et al., 2005). Briefly, 1-second (21 frames) interval mean red blood cell velocities were calculated using custom MATLAB software, generating a 2D gray scale plot of RBC location change with time. This enabled an unbiased quantification of RBC transit velocities within capillaries throughout the entire muscle. RBC velocity was determined from a total of 1658 one-second intervals in 115 uninjured
capillaries, and from 2269 one-second intervals in 138 neocapillaries, 28 days following femoral artery excision. Histograms of mean $V_{RBC}$ determinations from each uninjured capillary and neocapillary were generated to define the spatial heterogeneity of red blood cell velocity within the microvasculature. The number of non-flowing capillaries and neocapillaries was determined from blue light epi-illumination videos, cross-referencing with corresponding SAD images, evident as either empty channels of plasma or capillaries with stationary RBCs for at least 15 seconds duration.

**3.2.9 Assessment of Network Responsivity to Hypoxia**

To evaluate the network responsivity to an acute hypoxic challenge, I constructed a localized O$_2$ delivery system. The hindlimb and exposed EDL muscle was positioned in a custom-built frame (Sugru®, FormFormForm Ltd.) fashioned to stabilize the EDL muscle for an extended period ($\geq$8 minutes) of imaging, with a 2cm x 2mm opening. A custom-built gas flow chamber controlled by computer-modulated flow meters was integrated within the microscope stage (Ghonaim et al., 2011, Ellis et al., 2012). The hindlimb was positioned above the chamber with the EDL muscle contacting either an optically transparent O$_2$-permeable 100-µm thick polydimethylsiloxane (PDMS) polymer membrane or a 50-µm thick fluorosilicone acrylate disk (Ellis et al., 2012). Chamber O$_2$ levels were measured using a fiber-optic pO$_2$ sensor (Ocean Optics) in the gas outlet. Gas consisting of 12% O$_2$, 5% CO$_2$, and 83% N$_2$ at 37°C was passed through the chamber for 5 minutes to generate a hyperoxic surface O$_2$ environment across the entire EDL, and standardize the baseline O$_2$ content for both normal and regenerated muscle. This %O$_2$ was selected based on a previously established capillary normoxia tension in the rat EDL of 6.3% O$_2$ (Goldman, 2008). IVVM blood flow recording was initiated and 30 seconds later the EDL muscle was subjected to the hypoxia challenge by changing the gas content to 2% O$_2$, 5% CO$_2$, and 93% N$_2$, effectively removing O$_2$ from the EDL muscle. RBC transit was continuously recorded before and after the hypoxia challenge for a minimum of three minutes, and $V_{RBC}$ and RBC supply rate (#RBCs/sec) were quantified.

Mean $V_{RBC}$ and mean RBC supply rates were determined in a total of 255 capillaries from control and regenerated EDL muscles. Hyperoxia RBC measurements
were based on 30 seconds of recording prior to induction of hypoxia. Hypoxia RBC measurements were acquired from 120-180 seconds of recording following a 20 second transition phase. A stable hyperemic response to hypoxia was considered sustained if the plateau $V_{RBC}$ persisted for at least 120 seconds.

### 3.2.10 Laser Scanning Confocal Microscopy and 3-Dimensional Reconstruction of Precapillary Arterioles

For thick-section immunostaining, mice were sacrificed by isofluorane overdose and perfused sequentially with PBS and 4% PFA via left ventricle cannulation at physiologic pressure. EDL muscles were dissected, immersed in 4% PFA for 2 hours, cryoprotected with 15% sucrose for 2 hours and 30% sucrose overnight at 4°C. Tissues were then embedded in OCT embedding medium (Tissue-Tek) and stored at -80°C. 100-µm thick longitudinal cryosections were permeabilized with 0.5% Triton-X in PBS and double-immunostained using biotinylated rat anti-mouse CD31 antibody (1:50) and mouse anti-smooth muscle (SM) α-actin alkaline phosphatase-conjugated antibody (1:50, Clone 1A4, Sigma A5691). Bound antibodies were visualized using Streptavidin-488 (1:100, Sigma) and Alexa Fluor 546-conjugated goat anti-mouse IgG (1:200, Life Technologies). Nuclei were visualized with TO-PRO-3 iodide (1:500, Life Technologies). Thick-sections were transferred to positively charged glass slides, mounted with PermaFluor (Thermo Scientific), and flanked with 100-µm thick plastic coverslip spacers (Thermo Scientific) prior to coverslipping and sealing.

Arterioles 7-20 µm in diameter were imaged with an LSM 510 Meta Confocal Microscope (Zeiss) using a 40x water-immersion objective and Argon2 (488 nm excitation) and HeNe1 (543 nm excitation) lasers, generating up to 50, 0.5 µm-thick z-slices at 2048x2048 image resolution. Z-slices were reconstructed into 3-dimensional projections with Image Viewer (Leica) software. To quantify the spacing between adjacent SMC processes, the SM α-actin (red) signal was isolated from 3-dimensional projections with Photoshop (Adobe) and the maximum distance from one SM-α-actin containing process to the next along the arteriolar center-axis was ascertained using ImageJ. Vessel coverage by SMCs was determined after measuring the total inter-process
gap area and total arteriole segment area ImageJ.

3.2.11 Statistics

All values passing D’Agostino and Pearson omnibus normality testing were presented as mean ± SEM. Those not passing the normality test were presented as median and IQR. Comparisons among normally distributed variables were made by t-test or analysis of variance with Bonferroni’s post hoc test. Comparisons among hypoxia RBC measurements, width of inter-process gaps, and \( V_{RBC} \) were made by Mann-Whitney test or Kruskall-Wallis test with Dunn’s post hoc test. Comparisons among percent of arterioles with AV malformations, percent of capillaries depicting a greater than 5-fold hyperemic hypoxia response, and percent of capillaries sustaining a hyperemic hypoxia response were made by Fisher’s exact contingency test. Mean \( V_{RBC} \) histograms were compared using a two-way Kolmogorov-Smirnov test in MATLAB custom software.

3.3. Results

3.3.1 Femoral Artery Excision Induces Widespread Infarction and Capillary Obliteration in the EDL Muscle Followed by Extensive Regeneration

To study the regeneration of microvessels following ischemic injury, C57BL/6 mice were subjected to right femoral artery excision. I screened all muscles in the hindlimb for the extent of infarction and identified that the anterior muscle bundle distal to the knee was the most extensively damaged (data not shown). Within this bundle is the extensor digitorum longus (EDL) muscle, which I found to be 2 cm long, 2 mm wide, and with well-defined borders making it well suited to a comprehensive assessment. Furthermore, the EDL was accessibly located for live imaging. Histological assessment revealed that one day following femoral artery excision, the entire EDL muscle was infarcted and necrotic. Myofibers were shrunken, had lost their nuclei, and stained palely with eosin (Figure 3.1 a,b). However ten days after surgery the muscle had repopulated with intensely staining myocytes with large central nuclei, indicative of regenerated skeletal myocytes (Figure 3.1 c) (Paoni et al., 2002). By 28 days the regenerated myocytes had matured, with larger diameters and more condensed nuclei (Figure 3.1 d).
Figure 3.1 Histology of the EDL muscle before and after femoral artery excision

A-D. Hematoxylin and eosin-stained sections of EDL muscle before surgery (A), one day following surgery showing pale, shrunken myocytes without nuclei (B), after 10 days showing repopulated myocytes with central nuclei (arrows) (C), and after 28 days (D).
To evaluate the microvasculature, sections were immunostained using a panel of distinct endothelial markers. Immunostaining uninjured EDL muscles for CD31, endomucin, vWF, and isolectin-b4 revealed capillaries at the interface between the skeletal myofibers (Figure 3.2 a). One day after femoral artery excision, immunoreactivity relative to control muscle fell to 14%, 11%, 23%, and 44% for each marker, respectively (Figure 3.2 b). Remarkably, three days after surgery, immunoreactivity for CD31, endomucin, and vWF was entirely absent, with some residual isolectin-b4 staining (34%) (Figure 3.2 a,b). Notably, the latter stains basement membranes in addition to endothelial cells (Peters et al., 1979). Furthermore, H&E staining revealed a complete absence of capillaries, with only the occasional fibrous ghost structure without nuclei (Figure 3.3 a,b). Capillary obliteration was evident down to ~150 µm below the muscle surface with only scattered capillaries in the muscle core. Interestingly, neocapillaries emerged thereafter throughout the EDL muscle, in concert with the skeletal myogenesis (Figure 3.4 a). By 28 days their density exceeded that of control EDL muscle by 1.65-fold (P=0.006, Figure 3.4 a,b) although with a capillary-to-muscle fiber ratio that was similar to control muscle (1.09±0.06 vs. 1.07±0.06, P=0.841, Figure 3.4 b). Collectively, these findings reveal extensive capillary destruction in EDL muscle following ischemic injury, followed by a vigorous microvascular regeneration program that was associated with skeletal muscle regeneration.

3.3.2 The Regenerated EDL Muscle Displays Chronic Hypoxia Despite Robust Angiogenesis

I next asked whether the regenerated EDL muscle returned to a well-oxygenated state. To assess for hypoxia, tissues were immunostained for pimonidazole adducts following in vivo perfusion with Hypoxyprobe-1. Control EDL muscle showed no hypoxia signal (Figure 3.5 a). However, regenerated EDL muscle (28 days) displayed widespread hypoxia signals in the muscle fiber cytoplasm and nuclei, with myofiber-to-myofiber variability (Figure 3.5 b,c). I also identified increased expression of the hypoxia-dependent, HIF-1α-regulated genes adrenomedullin (P=0.004), lysyl oxidase (LOXL2) (P=0.043) and procollagen lysyl hydroxylase (PLOD2) (P=0.008, Figure 3.5 d). Interestingly, expression of VEGF-A, which can stabilize sooner than that of
Figure 3.2 Absence of EC markers in the EDL muscle following femoral artery excision

A. Fluorescence micrographs of EDL muscle before and 3 days after surgery immunostained for CD31, endomucin, vWF, and isolectin-b4 showing capillaries (arrow) surrounding myofibers (asterisk) in control EDL and loss of capillaries following ischemic insult. Nuclei are visualized with DAPI.

B. Quantitative data for capillary density as identified by each marker. (n=5 native; n=3 each for days 1 and 3, *p<0.0001, †p=0.001, ‡p=0.0002, §p=0.0013, ||p=0.0004 vs native).
Regenerated EDL Muscle Displays Chronic Hypoxia.

We next asked whether the regenerated EDL muscle returned to a well-oxygenated state. To assess for hypoxia, tissues were perfused with hypoxyprobe-1. Control EDL muscle showed no hypoxia signal (Figure 2A). However, regenerated EDL muscle (28 days) displayed widespread hypoxia signals in the muscle fiber cytoplasm and nuclei, with myofiber to myofiber variability (Figure 2B and 2C). We also identified increased angiogenic level of hypoxia.

To establish the network architecture of the regenerated microvasculature, we performed real-time microscopy after angiopoietin-1 (vascular endothelial growth factor-A), which can stabilize the regenerated EDL muscle was subjected to a chronic, non-angiogenic level of hypoxia.

Despite having a capillary–myofiber ratio similar to baseline, substantial upregulation of angiopoietin-1 (vascular endothelial growth factor-A), which can stabilize the microvasculature, was observed. Expression of VEGF-A (vascular endothelial growth factor-A), which can stabilize the microvasculature, was observed. Expression of VEGF-A and neither was that of procollagen lysyl hydroxylase was observed. However, angiopoietin-2 was substantially lower than in the control muscle (P = 0.0002, § P = 0.0004 vs native).

Graphs showing density of capillaries and capillary-to-myofiber ratios in native (n=3) and regenerated (28 days, n=3) EDL muscle (*P = 0.001, ‡ P = 0.006). These findings suggest that the microvasculature in the regenerated EDL muscle is not fully matured.

In Vivo Microvascular Imaging Reveals Robust Branching Architecture

Network Regeneration but With an Aberrant Quantitative data for capillary density as identified by each marker.

Photomicrographs of EDL fibers (asterisk) in control EDL and loss of capillaries after ischemic insult. Photomicrographs of EDL fibers (asterisk) in control EDL and loss of capillaries after ischemic insult.

**A**

![Image of immunostained sections of EDL muscle](image)

**B**

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*P < 0.0001, †P < 0.0001, ‡P < 0.0001, §P < 0.0001, ‖P < 0.0001.
Figure 3.3 Loss of capillaries following ischemic injury to the EDL muscle

A-B. Hematoxylin and eosin-stained sections of EDL muscle before (A) and three days following femoral artery excision (B). The native muscle fibers are surrounded by capillaries and discrete endothelial cell nuclei can be seen (A, arrows). Following ischemic injury, the skeletal myocytes have lost their nuclei. Capillaries are no longer evident, with only the occasional fibrous ghost structure without nuclei (B, arrowheads).
Online Figure I  Loss of Capillaries Following Ischemic Injury to the EDL Muscle

A-B. Hematoxylin and eosin-stained sections of EDL muscle before (A) and three days following femoral artery excision (B). The native muscle fibers are surrounded by capillaries and discrete endothelial cell nuclei can be seen (A, arrows). Following ischemic injury, the skeletal myocytes have lost their nuclei. Capillaries are no longer evident, with only the occasional fibrous ghost structure without nuclei (B, arrowheads).
Figure 3.4 Regeneration of capillaries in the EDL muscle following femoral artery excision

A. Photomicrographs of EDL muscle immunostained for CD31 showing capillaries in uninjured EDL muscle (left, arrows), their disappearance one day following surgery (middle), and regenerated capillaries 28 days following surgery (right, arrows).

B. Graphs showing density of capillaries and capillary-to-muscle fiber ratios in native (n=3) and regenerated (28 days, n=3) EDL muscle (*p=0.006).
Figure 3.5 Evidence for hypoxia in the regenerated EDL muscle

**A-B.** Photomicrographs of sections of native (A) and regenerated (day 28, B) EDL muscle harvested after infusion of Hypoxyprobe-1 and immunostained for pimonidazole.

**C.** Graph depicting quantified pimonidazole signal in regenerated EDL muscle not infused with Hypoxyprobe-1 (technical control, n=3), in native (contralateral) muscle (n=4), and regenerated muscle (n=4, *p=0.007).

**D.** Graph depicting relative mRNA abundance of hypoxia-dependent genes in uninjured (n=4) and regenerated (n=4) EDL muscles (*p=0.004, 0.043, 0.008, 0.029, 0.003 vs respective control).
Adrenomedullin (Fujita et al., 2002), was not greater than that in the control muscle (P=0.272) and neither was that of angiopoietin-1 (P=0.370). However, angiopoietin-2 was substantially upregulated (P=0.029) as was the ratio of angiopoietin-2/angiopoietin-1 (P=0.003). These findings suggest that, despite having a capillary-myofiber ratio similar to baseline, the regenerated EDL muscle was subjected to a chronic, non-angiogenic level of hypoxia.

3.3.3 In Vivo Microvascular Imaging Reveals Robust Network Regeneration But With An Aberrant Branching Architecture

To establish the network architecture of the regenerated microvasculature, I performed real-time microscopy following intravenous injection of FITC-labeled high molecular size Dextran. This enabled visualization of flowing microvessels, with RBCs in relief against the fluorescent plasma, within a 50 µm-thick zone along the entire EDL muscle. As well, I generated RBC transit maps, from 15-second video sequences, which revealed the location and geometry of all flowing microvessels, contrasted against stationary and thus non-visible muscle. Transit maps and live video sequences revealed a highly organized microvasculature in uninjured EDL muscle with hierarchical units of feeder arterioles, capillaries, and post-capillary venules (Figure 3.6 a, Online Video 3.1). Feeder arterioles penetrated orthogonally through the muscle to the surface zone, where they bifurcated into capillaries. Capillaries could also be seen to bifurcate, as they coursed parallel to the EDL surface, ultimately converging and draining into venules.

One day following femoral artery excision, blood flow in the EDL microvasculature had ceased and there was no RBC transit signal (Fig. 3.6 a, Online Video 3.2). However, 10 days after injury, an extensive but relatively chaotic network of flowing neovessels had regenerated (Figure 3.6 a). Quantitative analysis confirmed a hyper-vascular network, with a 21% greater length density than in normal EDL muscle (P=0.037, Figure 3.6 b) and a 3-fold increase in the number of branches, normalized to total network length (P<0.0001, Figure 3.6 c). Although microvessels of different lumen caliber were present, there was no evidence for arteriole-capillary-venular hierarchy at this time point (Online Video 3.3). However, the network subsequently underwent rapid
Figure 3.6 Microvascular network architecture before and after ischemic injury

A. RBC transit maps within native EDL muscle microvasculature and one, ten, 28, 56, and 120 days following femoral artery excision, showing no RBC transit one day after ischemic insult and subsequent regeneration of a flowing EDL microvasculature. Hatched lines denote EDL muscle borders. Corresponding real-time videos for each panel are available in the Online Supplement.

B-C. Graphs showing EDL muscle microvascular network length density (B) and branch density (C) (n=3-5 for each time point) B: *p=0.037, 0.041, 0.019, <0.0001, <0.0001, respectively vs control; C: *p<0.0001 vs control, † p<0.0001 vs. Day10.
structural maturation. Transit maps indicated that by day 14 there had been substantial branch pruning (Figure 3.6 c) and both network length and branch density stabilized by day 14-21. Real-time tracking of RBCs also revealed that the network had acquired arteriole-capillary-venule hierarchy by day 14.

Twenty-eight days after ischemic injury, the network resembled that of the uninjured EDL muscle (Figure 3.6 a, Online Video 3.4). Notably however, there was still a 21% greater vessel length density compared to the normal network (P=0.019) and a residual 1.6-fold increase in bifurcations (P<0.0001). Remarkably, elevated length and branch densities were still present 56 and 120 days after ischemic injury (Figure 3.6 a-c, Online Videos 3.5 and 3.6).

I also identified several abnormalities in architecture of the feeder arterioles. First, arterioles were seen to course parallel to the muscle fibers, rather than orthogonal to them, running up to 1 mm before bifurcating (Figure 3.7). Second, abnormalities in arterial branch caliber were found. In uninjured muscle, arterioles bifurcated into equal-caliber daughter branches whereas neo-arterioles branched into daughter vessels with strikingly unequal lumen diameters (Figure 3.7, Online Video 3.7). Third, neo-arterioles were seen not only to bifurcate, but also to trifurcate and quadrificate (Figure 3.7), a distinctly abnormal feature of the microcirculation (Fukumura et al., 2010, Less et al., 1991). Whereas only bifurcations were found in control muscle, supernumerary branching was identified in 6.5, 6.7, and 7.7% of arterioles within the neovasculature on days 28, 56, and 120, respectively.

I next asked how the altered architecture of the regenerated network might impact overall resistance to flow. To assess this, I undertook computational modeling of flow in the network, based on information derived from the videos and the processed RBC transit maps, for control and regenerated (28-day) networks. This predicted a 66% decline in network resistance, indicating a hemodynamic consequence of the altered network architecture (P=0.032, Figure 3.8). Thus, despite a rapid microvascular regeneration cascade, these findings reveal multiple architectural abnormalities, even in the late-stage network, that are predicted to impact hemodynamics within the network.
Figure 3.7 Aberrant architecture of regenerated feeder arterioles

Transit maps of branching arterioles of native and regenerated EDL muscle showing symmetrical bifurcation, asymmetrical bifurcation, trifurcation, and quadrification. Arrows depict the direction of parent vessel flow. The curved arrow denotes orthogonal inflow from below the plane.
RBCs Transit Slowly Through the Regenerated Microvasculature

We next sought to determine whether RBC transit through the regenerated network differed from that of normal EDL muscle. Within the uninjured EDL, RBCs traversed through capillaries in single file with a velocity ($V_{RBC}$) of 526.8 (337.0–727.0) μm/s (median [IQR]; Figure 4A). In contrast, within the early regenerated vasculature (10 days), median $V_{RBC}$ was only 36% of that of native capillaries ($P < 0.0001$). RBC transit was chaotic, with frequent direction changes as they passed through the hyperbranched, nonhierarchical network. Furthermore, the smallest caliber neovessels had 2 to 3 RBCs tumbling side-by-side, rather than transiting in single file (Online Movie III).

At 28 days, single file RBC transit in neocapillaries was evident, and arteriolar and venular flow was discernable (Online Movie IV). However, median $V_{RBC}$ remained low, at 52% of that of normal capillaries ($P < 0.0001$). Notably, $V_{RBC}$ was even lower on days 56 and 120 (23% of that of normal capillaries; $P < 0.0001$; Figure 4A).

We also identified an abundance of neocapillaries in which blood flow was halted altogether. This was evident either as a static column of plasma devoid of RBCs or as channels with stationary RBCs (Figure 4B; Online Movie VIII). On average, $2.0 \pm 1.6$ capillaries/mm$^2$ in the entire control EDL microvasculature displayed stopped flow for a duration of at least 15 seconds, and this increased by over 9-, 19-, and 19-fold in the regenerated EDL on days 28, 56, and 120, respectively (Figure 4C).

Regenerated Microvasculature Contains Arteriolar–Venular Malformations

The movement of oxygenated RBCs from arterioles to capillaries is fundamental to efficient oxygen delivery. However, we found that despite the return of single file capillary transit, not all arterioles diverged into a capillary network. Instead, we observed neoarterioles from which one branch diverged into a capillary mesh, while the other branch flowed directly into a venule (Figure 5A and 5B; Online Movies IX–XI). RBC transit within the direct arteriovenous connection was not single file, and flux was demonstrably greater than in the adjacent capillary system, revealing conditions for diverting oxygenated RBCs directly into the venule. Quantitative analysis revealed that 37%, 32%, and 33% of all neoarterioles on days 28, 56, and 120, respectively, branched into an arteriovenous connection, whereas arteriovenous shunts were rarely observed (2%) in control muscle ($P < 0.0001$; Figure 5C).

Figure 3. Microvascular network architecture before and after ischemic injury. A, Red blood cell (RBC) transit maps within native extensor digitorum longus (EDL) muscle microvasculature and 1, 10, 28, 56, and 120 days after femoral artery excision, showing no RBC transit 1 day after ischemic insult and subsequent regeneration of a following EDL microvasculature. Hatched lines denote EDL muscle borders. Corresponding real-time movies for each panel are available in the Online Data Supplement. B and C, Graphs showing EDL muscle microvascular network length density (B) and branch density (C; n=3–5 for each time point). B: *$P = 0.037$, 0.041, 0.019, <0.0001, and <0.0001, respectively, vs control; C: *$P < 0.0001$ vs control, †$P < 0.0001$ vs day 10. D, Transit maps of branching arterioles of native and regenerated EDL muscle showing symmetrical bifurcation, asymmetrical bifurcation, trifurcation, and quadri...
**Figure 3.8 Capillary network hemodynamic resistance**

**A-B.** Three-dimensional microvascular network reconstructions of IVVM data collected from native (A) and regenerated (B, 28 days) EDL muscle. Each reconstruction is a collection of nodes and cylindrical vessel segments. Color coding denotes depth separation of the capillary segments.

**C.** Graph showing capillary network hemodynamic resistance ascertained by simulating two-phase (RBCs and plasma) steady-state blood flow through three-dimensional network reconstructions (n=3 native and regenerated network reconstructions, *p=0.032).
Online Figure II  Capillary Network Hemodynamic Resistance

A-B. Three-dimensional microvascular network reconstructions of IVVM data collected from native (A) and regenerated (B, 28 days) EDL muscle. Each reconstruction is a collection of nodes and cylindrical vessel segments. Color coding denotes depth separation of the capillary segments.

C. Graph showing capillary network hemodynamic resistance ascertained by simulating two-phase (RBCs and plasma) steady-state blood flow through three-dimensional network reconstructions (n=3 native and regenerated network reconstructions, *p=0.032).
3.3.4 Red Blood Cells Transit Slowly Through The Regenerated Microvasculature

I next sought to determine if RBC transit through regenerated network differed from that of normal EDL muscle. Within the uninjured EDL, RBCs traversed through capillaries in single-file with a velocity ($V_{RBC}$) of 526.8 (337.0-727.0) µm/s (median (IQR), Figure 3.9 a). In contrast, within the early regenerated vasculature (10 days), median $V_{RBC}$ was only 36% of that of native capillaries (P<0.0001). RBC transit was chaotic, with frequent direction changes as they passed through the hyper-branched, non-hierarchical network. Furthermore, the smallest caliber neovessels had 2-3 RBCs tumbling side-by-side, rather than transiting in single-file (Online Video 3.3). At 28 days, single-file RBC transit in neocapillaries was evident and arteriolar and venular flow was discernable (Online Video 3.4). However, median $V_{RBC}$ remained low, at 52% of that of normal capillaries (P<0.0001). Notably, $V_{RBC}$ was even lower on days 56 and 120 (23% that of normal capillaries, P<0.0001, Figure 3.9 a).

I also identified an abundance of neocapillaries in which blood flow was halted altogether. This was evident either as a static column of plasma devoid of RBCs or as channels with stationary RBCs (Figure 3.9 b, Online Video 3.8). On average, 2.0±1.6 capillaries/mm$^2$ in the entire control EDL microvasculature displayed stopped-flow for a duration of at least 15 seconds and this increased by over 9-, 19-, and 19-fold in the regenerated EDL on days 28, 56, and 120, respectively (Figure 3.9 c).

3.3.5 The Regenerated Microvasculature Contains Arteriolar-Venular Malformations

The movement of oxygenated RBCs from arterioles to capillaries is fundamental to efficient oxygen delivery. However, I found that despite the return of single-file capillary transit, not all arterioles diverged into a capillary network. Instead, I observed neo-arterioles from which one branch diverged into a capillary mesh while the other branch flowed directly into a venule (Figure 3.10 a,b, Online Videos 3.9-3.11). RBC transit within the direct AV connection was not single-file and flux was demonstrably greater than in the adjacent capillary system, revealing conditions for diverting oxygenated RBCs directly into the venule. Quantitative analysis revealed that 37, 32, and
Figure 3.9 RBC transit in capillaries within the EDL microvasculature

A. Box and whisker (10-90 percentile) plot of red blood cell velocities \( V_{RBC} \) in individual capillaries within native and injured/regenerating EDL muscle evaluated at the designated times. Values were obtained from a total of 860 capillaries. Each value is derived from RBC velocities, averaged over a 15-second imaging time-frame, within each capillary.

B. Sequential video still-frames depicting dynamic RBC transit in control muscle (boxes, top row) and stalled perfusion in 28-day regenerated muscle (boxes, middle and bottom row). RBCs appear in relief against the fluorescent plasma. Arrows depict stagnant RBCs. Corresponding real-time videos are found in the Online Supplement (Online Video 3.8).

C. Graph depicting density of capillaries with flow halted for at least 15 seconds. \( n=4 \) native and \( n=3 \) for each of day 28, 56, and 120 EDL muscles.
For Circulation Research Peer Review. Do not distribute. Destroy after use.

**A**

![Graph showing Red Blood Cell Velocity (µm/s) over time](image)

**B**

*Dynamic RBC transit*

- 0 seconds (frame 1)
- 9 Seconds (frame 190)
- 18 seconds (frame 380)

*Stalled plasma column*

*Stalled RBCs and plasma*

**C**

![Bar graph showing Non-Flowing Capillaries per mm²](image)
Figure 3.10 RBC transit maps of arteriole-capillary-venule units

A. Native EDL muscle microvasculature with a parent arteriole that bifurcates into daughter vessels, both of which diverge into capillary meshes that drain into a venule system. A simplified, not-to-scale, schematic of the unit is shown on the right.

B. Regenerated EDL muscle (day 28) with a parent arteriole that trifurcates into daughter vessels of unequal caliber. Daughter vessel one diverges into a capillary mesh that drains into a venule whereas daughter vessel two drains directly into a venule, also depicted in the adjacent schematic. Corresponding real-time videos are found in the Online Supplement (Online Videos 3.9 and 3.10) and reveal high RBC flux in the AV connection.

C. Graph depicting the percentage of arterioles that branch into an AV connection. The entire network from 5 native EDL muscles and from 3, 4, and 4, regenerated muscles on days 28, 56, and 120, respectively was evaluated as indicated below the graph (*p<0.0001 vs native).
33% of all neo-arterioles on days 28, 56, and 120, respectively, branched into an AV connection, whereas AV shunts were rarely observed (2%) in control muscle (P<0.0001) (Figure 3.10 c).

3.3.6 RBC Velocity Dispersion is Severely Blunted in the Regenerated Microvasculature With An Abnormal Oxygen Delivery Profile

I next assessed for heterogeneity in RBC transit among capillaries within a network (Duling et al., 1987, Ellis et al., 1994). This heterogeneity is fundamental to the skeletal muscle circulation and critical for precisely matching the metabolic demands of each myofiber in the muscle with RBC delivery (Ellis et al., 2012, Ellis et al., 1994). To assess network heterogeneity, I compared the frequency distributions of mean \( V_{RBC} \) amongst capillaries over the entire EDL zone. The inter-capillary \( V_{RBC} \) histogram derived from the 28-day regenerated network was significantly different than that of the native network, with both a leftward shift and narrower range (K-S stat=0.54, \( P=1.12 \times 10^{-13} \)) (Figure 3.11). In addition, the normal microcirculation was found to contain a hyperemic subpopulation of capillaries with a particularly high \( V_{RBC} \) (>1000 \( \mu \)m/s). In contrast, there was no hyperemic subpopulation of capillaries in the regenerated network day 28. These abnormalities did not normalize over time and in fact were worse on days 56 and 120. Thus, the regenerated microcirculation displayed profoundly reduced dispersion in \( V_{RBC} \), suggesting an inability of the network to tune the delivery of \( O_2 \) to meet the needs of individual myofibers.

To determine if the homogeneity in \( V_{RBC} \) was associated with abnormal \( O_2 \) delivery, I directly measured hemoglobin \( O_2 \) saturation in RBCs transiting through the capillaries. Using dual wavelength absorption microscopy to distinguish oxy- and deoxyhemoglobin, I found that median RBC \( O_2 \) saturation in regenerated capillaries (120 days) was 75% of that in normal capillaries (\( P=0.0003 \)). This reduction was evident not only for RBCs at the downstream end of the capillary (69%, \( P=0.002 \)) but also at the upstream end (77%, \( P=0.005 \)) (Figure 3.12). Furthermore, there was a strikingly wide spread in RBC \( O_2 \) saturation in the regenerated capillaries, with \( O_2 \) saturations as low as 2% and as high as 87%. These findings indicate the variable presence of regions with
Figure 3.11 RBC velocity dispersion within the microvasculature

Frequency histograms of the average capillary VRBC within native and regenerated EDL muscles 28, 56, and 120 days after injury. The distribution is significantly narrowed on day 28 (K-S stat=0.54, p=1.12x10^{-13}) and remains narrow and left-shifted.
muscle through a gas-permeable membrane. After generating a stable $P_{O_2}$ of 12% at the muscle site, we abruptly decreased the ambient oxygen content to 2%, establishing conditions for microremoval of muscle $O_2$. The ability of the microvasculature to respond to this hypoxic challenge was then evaluated by tracking both RBC velocity and RBC supply rate through individual capillaries.

Figure 6B depicts RBC velocity response profiles to local hypoxia, illustrating a brisk and sustained $V_{RBC}$ increase in a native capillary but a blunted and transient response in a regenerated capillary. Although there was a range of responses among capillaries (Figure 6C and 6D), overall, there was a significant reduction in both $V_{RBC}$ and RBC supply rate responsivity within the regenerated microvascular network. Among 163 capillaries in normal EDL muscle, hypoxia induced a 1.53-fold increase in median $V_{RBC}$ and a 1.7-fold increase in median RBC supply rate (Figure 6C and 6D; Online Movie XII). Relative to these responses, the $V_{RBC}$ and RBC supply rate responses in the regenerated capillaries were significantly lower than in the native capillaries.

Figure 6. Red blood cell (RBC) velocity dispersion within the microvasculature and network responsivity to hypoxia. A, Frequency histograms of the average capillary RBC velocity ($V_{RBC}$) within native and regenerated extensor digitorum longus (EDL) muscles 28, 56, and 120 days after injury. The distribution is significantly narrowed on day 28 ($K-S$ stat=0.54; $P=1.12 \times 10^{-13}$) and remains narrow and left-shifted. B, RBC velocity response profiles to local hypoxia. $V_{RBC}$ were acquired every second within a single capillary for each condition before and after hypoxia challenge. C and D, Graphs depicting the $V_{RBC}$ and RBC supply rate responses to local hypoxia. Left, The fold-change response in individual capillaries is shown for day 28. The line depicts the median. Right, The relative median responses (and 45–55 percentile) at the time points indicated are shown. Data are from 164, 65, 27, and 60 capillaries from control (n=6), day 28 (n=3, day 56 (n=3), and day 120 (n=3) EDL muscles, respectively. For $V_{RBC}$: *$P=0.004$, †$P=0.001$, ‡$P=0.021$ vs control. For RBC supply rate: *$P=0.004$, †$P=0.0004$, ‡$P=0.005$ vs control.
**Figure 3.12 Capillary RBC hemoglobin O₂ saturation**

**A.** Box and whisker (min-max) plot of capillary RBC hemoglobin O₂ saturation in native capillaries in control EDL muscle and regenerated capillaries in EDL muscle 120 days after ischemic injury. Data are from 24 capillaries from 3 native EDL muscles and 56 neocapillaries from 3 regenerated muscles. *p=0.0003.

**B-C.** Plots depict RBC hemoglobin O₂ saturation selectively in post-arteriolar capillary segments (*p=0.005) and in pre-venular capillary segments (*p=0.002).
Online Figure III: Capillary RBC hemoglobin O\textsubscript{2} saturation

**A.** Box and whisker (min-max) plot of capillary RBC hemoglobin O\textsubscript{2} saturation in native capillaries in control EDL muscle and regenerated capillaries in EDL muscle 120 days after ischemic injury. Data are from 24 capillaries from 3 native EDL muscles and 56 neocapillaries from 3 regenerated muscles. *p=0.0003.

**B and C.** Depict RBC hemoglobin O\textsubscript{2} saturation selectively in post-arteriolar capillary segments (*p=0.005) and in pre-venular capillary segments (*p=0.002).
3.3.7 The Regenerated Microvasculature Has Impaired Flow Responsiveness to Hypoxia

To directly determine if the regenerated microcirculation could regulate blood flow, I generated a methodology to locally control the O₂ content in the mouse EDL muscle. A defined O₂-containing gas mixture was delivered to the EDL muscle through a gas-permeable membrane. After generating a stable PO₂ of 12% at the muscle site, I abruptly decreased the ambient oxygen content to 2%, establishing conditions for micro-removal of muscle O₂. The ability of the microvasculature to respond to this hypoxic challenge was then evaluated by tracking both RBC velocity and RBC supply rate through individual capillaries.

Figure 3.13a depicts RBC velocity response profiles to local hypoxia, illustrating a brisk and sustained $V_{RBC}$ increase in a native capillary but a blunted and transient response in a regenerated capillary. Although there was a range of responses among capillaries (Figure 3.13 b,c), overall there was a significant reduction in both $V_{RBC}$ and RBC supply rate responsivity within the regenerated microvascular network. Among 163 capillaries in normal EDL muscle, hypoxia induced a 1.53-fold increase in median $V_{RBC}$ and a 1.7-fold increase in median RBC supply rate (Figure 3.13 b,c, Online Video 3.12). Relative to these responses, the $V_{RBC}$ and RBC supply rates were significantly lower on each of days 28, 56, and 120 after ischemic injury (Figure 3.13 b,c). These findings identify that the regenerated microvasculature is impaired in its ability to effectively augment RBC delivery when faced with local hypoxia.

3.3.8 Smooth Muscle Cell Wrapping Around Regenerated Arterioles is Aberrant

The combination of impaired responsivity to hypoxia and the homogeneity of RBC velocities throughout the network implicated failure of a vasomotor tuning mechanism. I considered that a unifying basis for this failure might reside in the cellular architecture of the feeder arterioles. To elucidate the microstructure of normal and
**Figure 3.13 Network responsivity to hypoxia**

**A.** RBC velocity response profiles to local hypoxia. $V_{RBC}$ were acquired every second within a single capillary for each condition before and after hypoxia challenge.

**B-C.** Graphs depicting the $V_{RBC}$ and RBC supply rate responses to local hypoxia. On the left, the fold-change response in individual capillaries is shown for day 28. The line depicts the median. On the right, the relative median responses (and 45-55 percentile) at the time points indicated are shown. Data are from 164, 65, 27, and 60 capillaries from control (n=6), day 28 (n=3), day 56 (n=3), and day 120 (n=3) EDL muscles, respectively. For $V_{RBC}$: *p=0.004 †p=0.001, ‡p=0.021 vs control. For RBC supply rate: *p=0.004 †p=0.0004 ‡p=0.005 vs control.
regenerated arterioles, I generated 3-dimensional confocal reconstructions from 100 µm-thick sections of EDL muscle double-immunolabeled for CD31 and SM-α-actin. In normal EDL, arterioles were found to be intimately invested by SMCs. The precise architecture varied with arteriolar caliber and location within the tree. SMCs on arterioles of diameter 12-20 µm enveloped the artery with circumferential processes containing SM-α-actin, which I could resolve as individual microfilament bundles (Figure 3.14 a,b). SMCs on arterioles 7-12 µm diameter also circumferentially wrapped the vessel with SM-α-actin cytoplasmic protrusions, but these processes were somewhat thinner and more variably oriented (Figure 3.14 a,c). SMC investment and protrusion-based wrapping terminated once the vessel diameter fell to 5-8 µm, consistent with transition to a capillary (Figure 3.14 a).

SMCs were also found to layer around arterioles of the regenerated network and send out processes. However in contrast to native arterioles, the coverage was discontinuous and disordered (Figure 3.14 a-c). In some neo-arterioles, there were zones up to 13 µm in length that were largely devoid of SM-α-actin bundles. Moreover even in regions where SMC processes wrapped the vessel, the spacing between adjacent processes was increased compared to that of normal arterioles of similar caliber (Figure 3.14 b,c arrows). Quantitative analysis revealed that the median space from one SM-α-actin containing process to the next in normal terminal arterioles was 0.74 µm (Figure 3.14 d). These gaps constituted 7% of the arteriolar surface area (Figure 3.14 e). In contrast, 28 days after injury the median inter-process space was 1.40 µm (P<0.0001), which corresponded to 22% of the neo-arteriolar surface area (P<0.0001, Figure 3.14 d,e). Increased process spacing was also observed at days 56 and 120, although this improved somewhat. However, prominent gaps in SMC process coverage were still evident, with widely variable process orientations, rendering a haphazard and disordered coverage profile (Figure 3.14 c, arrows). These findings strongly implicate arteriolar wall micro-disorder as a basis for impaired control over RBC delivery through the regenerated skeletal muscle.
Figure 3.14 Aberrant wrapping of SMCs around arterioles in the regenerated microvasculature

A. Confocal micrographs of distal arteriolar trees from normal and regenerated EDL muscle, immunostained for CD31 (green) and SM-α-actin (red). Images are projections of 30 optical sections. Arrows indicate sites of discontinuous SMC coverage.

B. Confocal micrographs of projected optical sections of arteriolar segments ~14-15 µm in diameter of control and regenerated (day 28) muscle, immunolabeled for SM α-actin (red).

C. Projected optical sections of arteriolar segments ~9 µm in diameter of control and regenerated (day 56 and 120) muscle, immunolabeled for SM-α-actin (red) and TO-PRO-3 (blue). Arrows indicate sites of discontinuous SMC coverage, and arrowhead indicates site of aberrant process orientation.

D. Graph depicting the SMC inter-process gap widths. Data are from 25 arterioles from native EDL muscles, and 23, 19, 22 neoarterioles from EDL muscles 28, 56, and 120 days after ischemic injury. *p<0.0001 vs native).

E. Graph depicting surface area coverage of feeder arterioles by SM-α-actin-containing processes. *p<0.0001 vs native.
3.4 Discussion

Through high-resolution mapping of RBC transit, dynamic tracking of RBC velocities, and optical reconstruction of terminal arterioles, I have discovered that the reconstructed microvasculature in EDL muscle is a highly-flawed network. The array of abnormalities I uncovered in this regenerated tissue included: 1) aberrant arteriolar branching; 2) arteriolar-venular shunting; 3) slow RBC transit through capillaries; 4) a monotony of RBC transit velocities throughout the capillary system; and 5) an impaired vasomotor control system. These abnormalities were still evident 4 months following injury, indicating a failure to normalize. The functional importance of these abnormalities was supported by the presence of hypoxia signals and profoundly abnormal capillary O$_2$ saturations in the EDL muscle, despite a capillary-to-skeletal myocyte ratio that was similar to that of normal EDL muscle. Thus, notwithstanding an extensive regenerative response, the microvascular neo-network was ill suited for effective oxygen delivery.

This is the first study to evaluate regenerative angiogenesis in skeletal muscle at a network level and at the site where gas and nutrient exchange occurs. The mouse EDL subjected to femoral artery excision proved to be a valuable model for this because the entire EDL muscle was infarcted and the native microcirculation was destroyed, as evidenced by staining with H&E and a panel of endothelial markers. All subsequently identified microvessels could thus reliably be attributed to a regeneration program. This contrasts with other sites in the mouse hindlimb where infarction is patchy and a mix of pre-existing and new microvessels would co-exist (Couffinhal et al., 1998, Helisch et al., 2006). High-resolution mapping of RBC transit in the regenerating EDL revealed that the vascularization response was in many respects impressive. It entailed an early hyper-vascular phase that covered the entire EDL muscle surface zone. Branching was extensive at this early stage and the network was not yet hierarchical. However, rapid branch pruning and re-alignment of microvessels quickly ensued, yielding a network that coursed primarily parallel to long axis of the maturing skeletal myofibers. Moreover, within 14 days of the ischemic insult, these vessels had differentiated into arteriole-capillary-venular circulation units, further indicating the rapid angiogenesis and network remodeling program.
Nevertheless, discordance between the microvascular restoration process and functionality of the RBC delivery system was remarkable. Whether the identified array of microcirculatory defects exists in all models of ischemia, as well as different mouse strains and forms of injury, is unknown. However femoral artery excision in C57BL/6 mice constitutes a substantial insult and is also a setting where the return of bulk flow is robust, reaching 85-100% that of the contralateral limb (Couffinhal et al., 1998, Scholz et al., 2002, Helisch et al., 2006). The current findings therefore highlight the “hidden” pathophysiology that can exist in the microcirculation. Some differences in capillary network architecture might be expected, given the different size and arrangement of regenerated myofibers. However, the abnormal RBC transit, impaired vasoreactive control, and violations of arteriole-capillary-venule geometry and hierarchy constitute previously unrecognized pathologies in this regenerative setting. Moreover, these defects would not be detectable with currently employed techniques for assessing blood flow, such as laser-Doppler, Doppler-ultrasound, and MR imaging strategies.

Our findings of asymmetric arteriolar bifurcations, trifurcations, and quadrifications in the regenerated microvasculature identify a distinctly abnormal arteriolar morphogenesis program. Bifurcation of arterioles into symmetrical caliber branches is fundamental to the microcirculation and ensures ordered dispersion of red cells though the capillary network (Koller et al., 1987, Al-Khazraj et al., 2015, Pries et al., 1989, Frame et al., 1993). Although the molecular underpinnings of this arteriolar dysgenesis response are unknown, it is noteworthy that both asymmetric arteriolar branching and super-numerary branching are features of the highly disordered microvasculature of tumors (Fukumura et al., 2010, Less et al., 1991). The similarities between the tumor and regenerated muscle milieu would only be partial but it is noteworthy that aberrant arteriole branching in tumors is considered to be one of the causes of tumor hypoxia (Fukumura et al., 2010, Less et al., 1991, Baish et al., 2000). The potential for arteriolar asymmetry to similarly contribute to hypoxia in skeletal muscle is also supported by mathematical modeling, which has indicated skimming of plasma, disparate local hematocrits, and regional heterogeneity in oxygen tensions unrelated to tissue needs (Less et al., 1991, Pries et al., 1989, Pries et al., 1996).
Our discovery of arteriole-venule shunts in the regenerated microcirculation was striking and reveals another basis for impaired oxygen delivery to the muscle. These aberrant conduits were not found in any of the uninjured muscles yet constituted the output pattern of 37% terminal arterioles. Their presence in muscle can be expected to compromise gas exchange by diverting red cells away from nearby single-file transit capillaries into the high flow micro-shunts. Interestingly, these micro-shunts were scattered throughout the vascular tree, which differs from the focal arterio-venous malformations seen in conditions such as hereditary hemorrhagic telangiectasia (Park et al., 2009, Braverman et al., 1990). The shunts emerged at the same time as capillaries were forming elsewhere in the network, suggesting a primary, albeit localized, failure in capillary differentiation. This differs from recent studies in the brain where arterial-venous malformations arise via pathological transformation of pre-existing capillaries (Murphy et al., 2014).

The velocity of RBCs within capillaries provides important clues about oxygen delivery in tissues. The sluggish transit of RBCs through regenerated capillaries was particularly noteworthy given that the metabolic demands of regenerating skeletal myofibers would be expected to increase, not decline (Koopman et al., 2014). Our transit maps and video analyses point to several causes of the slow capillary flow, including a greater total path length of the regenerated microvascular network and the steal phenomenon from the abundant arterio-venule shunts (Ellis et al., 2002, Lam et al., 1994). The persistent increase in the prevalence of capillaries with entirely halted RBC transit also suggests luminal plugging of microvessels and/or competing pressure heads within the network. The absence of capillaries in which RBC velocities rose above 1000 µm/s is also noteworthy because RBCs transiting through hyperdynamic capillaries have the potential to deliver oxygen not only to the adjacent tissue but to RBCs in nearby capillaries, effectively re-loading them as they approach the distal capillary segment (Ellis et al., 2012, Koning et al., 2014). Collectively, these abnormalities in RBC transit velocities strongly suggest that slow RBC transit is a pathological entity of the regenerated microvascular network.

The normal microvasculature does not act as an inert set of micro-tubes but,
instead, as an exquisitely controlled vasomotor system that distributes RBCs in an orchestrated manner to meet the needs of the tissue (Segal, 2005). Hypoxia is a powerful stimulus for enhancing delivery of RBCs in skeletal muscle (Jia et al., 2011, Parthasarathi et al., 1999). The oxygen-exchange strategy I employed, coupled with direct tracking of RBC transit in individual capillaries, provided a uniquely rapid means of generating a hypoxic muscle milieu and directly linking this to network responsivity. Whereas RBC velocity and supply rates in control EDL muscle rapidly increased in response to hypoxic challenge, the responses in the regenerated vasculature were blunted. Thus, the regenerated microvasculature was defective in a fundamental control process for modulating RBC delivery. This muted responsivity could also explain the striking monotony in RBC velocities I observed throughout the network. Although it is possible that the demands of the regenerated EDL were more uniform than in the normal EDL, this seems unlikely given the striking variations in capillary RBC $O_2$ saturation that I identified. Together, the findings indicate that the regenerated network does not have the ability to tune the delivery RBCs to match the regional needs.

What might be the mechanism for this relative inertness in the regenerated microcirculation? Distal arterioles constitute the critical effector limb for RBC delivery and the site where hormonal, metabolic, or neural regulatory factors impinge. Three-dimensional reconstruction of confocal optical images afforded an unprecedented assessment of how SMCs and their contractile elements are organized around these arterioles. Our finding of circumferential wrapping of SMC processes is consistent with studies using scanning electron microscopy (Holley et al., 1983) and has recently been delineated in brain arterioles using genetically encoded mural cells (Hill et al., 2015). Remarkably, in addition to the banded pattern of the SMC processes, I was also able to resolve individual actin microfilament bundles, which revealed there to be 3 to 5 discrete actin microfilament bundles per process, in 10-20 µm diameter arterioles. The finding that SMC processes also wrapped arterioles in the regenerated network is consistent with our intravital microscopy findings of arteriole-capillary-venule units. However, the increased spacing between the SM-α-actin containing processes identifies an architectural defect in the contractile machinery. In fact, in the regenerated vessels I found that there was more gap between the SM-α-actin containing processes than area covered by the
processes. This defect was even more striking in arteriolar segments immediately upstream of the capillary, where the actin processes were neither circumferential nor spiral, but oriented haphazardly. Given that arterioles of the caliber studied (7-20 µm) were invested by only a single layer of SMC processes, the architectural details of these processes stand to be vital determinants of the extent to which RBC entry into the capillaries is controlled. I do not currently know if additional defects in the vasomotor control loop exist, such as in oxygen sensing by the endothelium, gap-junction mediated signal conduction, or communication between the endothelium and SMCs. Nonetheless, the findings implicate an entirely new reason for microvascular dysfunction, based on disorder of the otherwise exquisitely patterned actin-containing protrusions.

In summary, using 4D microvascular imaging and an obliteration-restoration model of angiogenesis, I have uncovered profound and persistent network-level dysfunction in the regenerated microcirculation in mouse skeletal muscle. These findings suggest that, in the setting of vascular regeneration following skeletal muscle infarction, impaired network functionality rather than limited angiogenesis may be a key determinant of ongoing ischemia. Strategies to impart advanced physiology to the post-infarction microcirculation, including recapitulating normal arteriolar morphogenesis, are warranted.

3.5 References


CHAPTER 4

Vascular Regeneration Following Skeletal Muscle Ischemia
Occurs By Intussusceptive Angiogenesis and Differential
VEGFR2 Responsiveness

4.1 Introduction

Skeletal muscle is the largest organ in humans representing 40% of body weight and receiving 20-90% of total cardiac output (Pederson et al., 2012, Delp et al., 2004). The high and variable metabolic requirements of skeletal muscle demand an immense and finely tuned microcirculation, capable of regulating blood flow over a tremendous range (Segal, 2005). The microcirculatory architecture is that of a hierarchical network. Within this network, capillaries are strategically positioned at the interface of individual muscle fibers to allow RBCs to effectively deliver their oxygen (Krog, 1919, Arpino et al., 2017).

Skeletal muscle has a strong innate neovascularization response to ischemic injury (Couffinhal et al., 1998, Limbourg et al., 2009). One model that has served as an important platform for understanding neovascularization in skeletal muscle is excision or ligation of the femoral artery in mice (Limbourg et al., 2009). This model has demonstrated that, following ischemic injury, blood flow is naturally restored to skeletal muscle over time. Flow restoration occurs via the opening of collateral vessels and the regeneration of new microvessels (Couffinhal et al., 1998, Limbourg et al., 2009). Furthermore, several studies have demonstrated that this neovascularization response can be enhanced via exogenous growth factor and cellular delivery (Takeshita et al., 1994, Isner et al., 1996, Isner et al., 1999, Kalka et al., 1999). This regenerative response has formed the rationale for promoting “therapeutic angiogenesis” in the limbs of patients with peripheral arterial disease (PAD).
However, PAD remains a widespread clinical problem. Innate neovascularization appears to be limited or ineffective within the ischemic muscles of patients (Annex, 2013). In addition, therapeutically enhancing this process using strategies effective in mouse models has not been found to improve perfusion in the limbs of patients with PAD (Annex, 2013, Simons et al., 2003). Together, these limitations suggest that the current understanding of the cellular mechanisms and processes underlying neovascularization in ischemic skeletal muscle is incomplete.

Currently, the main tenets of adult neovascularization revolve around the process of sprouting angiogenesis (Eilken et al., 2010). Sprouting angiogenesis has been extensively studied in the developing mouse retina and the zebrafish embryo. The sprouting process is characterized by the protrusion and migration of endothelial tip cells from pre-existing vessels into surrounding tissue. Sprouting angiogenesis is substantially controlled by the VEGF signaling system. Tip cell selection requires VEGFA-VEGFR2 signaling, with subsequent downstream Dll4-Notch1 signaling (Ruhrberg et al., 2002, Gerhardt et al., 2003, Hellstrom et al., 2007). Sprouting angiogenesis has been identified in healthy adult skeletal muscle in response to mechanical stretch or chronic muscle contraction, on the basis of electron microscopic evidence for capillary endothelial cell (EC) abluminal protrusion (Egginton et al., 2001, Hansen-Smith et al., 1996, Zhou et al., 1998b).

Studies of hindlimb ischemia have consistently identified a robust increase in capillary density within ischemic skeletal muscle. This response has generally been attributed to sprouting angiogenesis and postnatal vasculogenesis (Hershey et al., 2001, Madeddu, 2005, Limbourg et al., 2009). The latter process has been reported to be driven by progenitor cells, either from bone marrow or within the ischemic skeletal muscle (Asahara et al., 1999, Asahara et al., 1997, Aicher et al., 2007, Leroyer et al., 2009, Ziegelhoeffer et al., 2004). However, for sprouting angiogenesis, there is remarkably little evidence to directly implicate its involvement in skeletal muscle angiogenesis following ischemic injury. To the best of our knowledge, only one study has directly identified an endothelial tip cell within the ischemic mouse skeletal muscle, in this case by immunostaining the injured adductor muscle (Al Haj Zen et al., 2010).
An additional mode of neovascularization and capillary generation has been identified and termed intussusceptive angiogenesis (IA). Intussusceptive, or splitting, angiogenesis entails the internal division of a single vessel into two distinct channels (Styp-Rekowska et al., 2011). IA has been identified in healthy skeletal muscle in response to elevated blood flow and high shear stress (Egginton et al., 2001, Zhou et al., 1998a, Hansen-Smith et al., 1996, Williams et al., 2006a, Williams et al., 2006b, Haas et al., 2000, Prior et al., 2004, Brown et al., 2003). One study has also reported IA in response to substantial overexpression of VEGF (Gianni-Barrera et al., 2013). However, IA in muscle is not known to be extensive and, relative to sprouting angiogenesis, the cellular and molecular processes regulating IA are poorly understood. Furthermore, IA has not been identified in ischemic skeletal muscle.

Herein, I describe the early events of de-novo microvascular network regeneration in skeletal muscle following ischemic injury. Using a real-time strategy for dynamically imaging RBCs within ischemic skeletal muscle (Arpino et al., 2017), I discovered that following ischemia-induced muscle infarction an unusual, dilated, and non-differentiated microvasculature with low blood flow and shear stress developed. This primordial microvasculature then rapidly transformed into an arborized network via EC “inward” protrusion and IA. Inward protrusion and primordial vessel splitting were associated with heterogeneity in EC phenotype and depended on suppressed VEGFR2 activation. These events prompt the reconsideration of angiogenesis paradigms in injured muscle and associated therapeutic strategies for ischemic disease.

4.2 Methods

4.2.1 Hindlimb Ischemia

Experiments were conducted in accordance with the University of Western Ontario’s Animal Care and Use Subcommittee. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) 12 weeks of age were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Hindlimb ischemia was
induced by ligating the right femoral artery above and below the profunda femoris branch using 6-0 silk sutures and excising the intervening 5-6 mm portion of artery (Limbourg et al., 2009, Frontini et al., 2011).

4.2.2 Histology and Immunostaining

The hindlimb anterior muscle bundle that includes the extensor digitorum longus (EDL), tibialis anterior, and peroneus longus muscles were dissected, fixed in either Tris-buffered zinc or 4% paraformaldehyde (PFA) overnight, and embedded in paraffin. Five-µm thick cross-sections of muscle through the proximal, middle, and distal regions were stained with hematoxylin and eosin. For immunofluorescence detection of ECs, near adjacent five-µm thick sections of muscle were subjected to heat-mediated antigen retrieval with citrate buffer and immunostained using rabbit polyclonal anti-mouse CD31 antibody (1:100, Thermo Scientific, RB-10333-P1) and detected using biotinylated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch) and Dylight 549-conjugated streptavidin (1:200, Vector) or rat monoclonal anti-mouse CD31 antibody (1:20, Dianova, Clone SZ31) and detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:100, Life Technologies). ECs were also immunostained using rat monoclonal anti-mouse endomucin antibody (1:100, Santa Cruz, Clone V.7C7, sc-65495) and detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:200, Life Technologies) or goat polyclonal anti-mouse VE-Cadherin antibody (1:100, R&D Systems, AF1002) and detected using Alexa Fluor-488 conjugated donkey anti-goat IgG (1:200, Life Technologies). For immunofluorescence detection of VEGFR2 activation in ECs, five-µm thick sections of muscle were subjected to antigen retrieval and double-immunostained using rat monoclonal anti-mouse endomucin antibody (1:100, Santa Cruz, Clone V.7C7, sc-65495) and rabbit polyclonal anti-VEGF receptor 2 phospho y1054 + y1059 antibody (1:50, Abcam, ab5473). Bound antibodies were detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:200, Life Technologies) and Alexa Fluor-594 conjugated goat anti-rabbit IgG (1:100, Life Technologies). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech, 0100-020). Sections were imaged by widefield microscopy (Olympus BX-51) and photomicrographs captured with Northern Eclipse (EMPIX Imaging Inc.) software.
4.2.3 Intravital Video Microscopy

RBC transit in individual microvessels within a 50 µm-deep zone across the entire mouse EDL surface was assessed by epifluorescent intravital video microscopy (IVVM) as described previously (Arpino et al., 2017). Briefly, mice were anesthetized with ketamine and xylazine intraperitoneally and a longitudinal incision was made over the anterior hindlimb. Tibialis anterior and peroneus longus muscles were separated from surrounding fascia and spread apart to reveal the underlying EDL (Tyml et al., 1991). The EDL was covered with an 8x8 mm glass coverslip, and positioned face-down on the stage of an inverted microscope (Olympus IX81), maintaining body temperature at 37°C. After a 20-minute stabilization period, the EDL was epi-illuminated with a 120 W Mercury X-Cite high-pressure bulb light source, via 10x and 20x objectives (Olympus UPlanSApo). RBC transit was visualized by either ultraviolet light epi-illumination (DAPI U-MWU2: 330-385 nm excitation filter, 420 nm emission filter) or blue light epi-illumination (U-MWIBA2: 460-490 nm excitation filter, 510-550 nm emission filter) (Frontini et al., 2011, Yin et al., 2015) following intrapenile injection of FITC-labeled dextran (2x10^6 MW; 20 mg/mL, 30 µL, Sigma). Video recordings (696 x 520 pixels, 21 images/sec) were captured using a cooled charge-coupled device camera (Rolera-XR, QImaging) and displayed in real-time on a computer monitor for at least 315 images (15 seconds). For all studies, the entire EDL surface was recorded and analyzed (7-10 fields of view). Video sequences were digitized and stored as uncompressed AVI files for post-processing using custom acquisition software (NeoVision) and in-house software written in the MATLAB (Mathworks) programming environment.

4.2.4 Analysis of Microvascular Network Architecture and Identification of Intraluminal Micro-Obstructions

Vessel lumen diameter, vascular density, bifurcation density, pillar density, and vessel split density of EDL muscle microvascular networks were quantified from RBC transit maps using ImageJ (NIH). RBC transit maps effectively displayed a microangiogram of all flowing microvessels, and were generated from IVVM video sequences using custom MATLAB software as described (Japee et al., 2004, Varghese et al., 2005).
From the videos generated by blue-light epi-illumination, RBC transit maps were generated from “sum of all differences” (SAD) images, which displayed the cumulative sum of the square of differences in light intensity values at each pixel between consecutive video frames, thus generating a single map of all microvessels perfused with RBCs and plasma.

Vessel lumen diameter was quantified manually perpendicular to the vessel centerline. Vascular density (microvasculature length [µm] / vascularized EDL area [µm²]) was quantified at each time-point via manual tracing of all vessel centerlines and normalizing total microvasculature length to vascularized EDL area. Bifurcation density was determined by manual point counting of all vessel bifurcations and normalizing to total microvasculature length. Intraluminal pillars were identified in RBC transit maps as circular (2-8 µm diameter) signal voids within flowing microvessels. Vessel splits were identified as RBC transit loops, wherein RBCs in transit diverged from a single vessel into two distinct smaller vessels and then reconverged into a single conduit at least 50 µm downstream. Pillar or split density was expressed as the total number of intraluminal pillars or vessel splits normalized to total microvasculature length. Arteriole-capillary-venule (ACV) microcirculatory units were identified from blue light epi-illumination videos and corresponding SAD images.

4.2.5 Cell Culture

Human umbilical vein endothelial cells (HUVECs, Lonza) were maintained in Endothelial Growth Medium-2 (EGM-2) BulletKit (Lonza, CC-3162) containing 2% fetal bovine serum and VEGF-A, with media changes every other day. After 8-10 passages, HUVEC cultures were generated at 70-75% confluence and were transfected with either control siRNA (50 nM, Origene, SR30004) or KDR (VEGFR2) siRNA (50 nM, Origene, SR302557) with Lipofectamine RNAiMAX kit (Thermo Fisher, 13778100) for 24 hours at 37°C. Six to eight hours following siRNA transfection, HUVECs were transduced with adenovirus encoding either RFP (5 µL/mL of media) or GFP (50 µL/mL of media) for 16-18 hours at 37°C. Approximately 32 hours following infection with siRNA, HUVECs were split and plated on glass coverslips at a 1:10 ratio (8000 siVEGFR2 HUVECs :
72000 siControl HUVECs) per well. Cells were grown for 48 hours to form a co-cultured monolayer at ~80% confluence. Cells were then fixed with 4% PFA for 20 minutes. Select monolayers were treated with S1P (1 µM in 0.3 M NaOH, Cayman Chemical, #62570) or vehicle solution for 3 minutes to stimulate activation of the cytoskeleton before fixation (Vouret-Craviari et al., 2002). VEGFR2 knockdown was confirmed following cell harvest and lysing by quantitative RT-PCR and SYBR Green chemistry and a ViiA 7 Real Time PCR System (Life Technologies). Primer pairs (Origene) employed were 5’- GGAACCTCACTATCCGCAGAGT-3’ and 3’- CCAAGTTCTCTTTCTCCTGGGC-5’.

4.2.6 Whole-Mount Immunostaining of Mouse Retinas

For whole-mount immunostaining of mouse retinas, retinas were harvested from five day-old C57BL/6 mice as described (Pitulescu et al., 2010). Briefly, mice were sacrificed by isoflurane overdose and eyeballs were enucleated. Eyeballs were rinsed in PBS, then fixed with 4% PFA for four hours at 4°C. After dissection the retina was rinsed briefly with PBS, and incubated with retina-blocking buffer (RBB) (PBS pH 7.4, 0.5% Triton-X 100, 1% BSA, 5% Donkey Serum) overnight at 4°C. The retina was then washed with Pblec buffer (PBS pH 6.8, 1% Triton-X-100, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mM MnCl₂) and incubated with biotynilated isolectin-b4 (1:50, Sigma, BSI-B₄, L2140) in Pblec buffer overnight at 4°C. Next, the retina was washed with 1:1 RBB:PBS, and incubated with rabbit polyclonal anti-VEGF receptor 2 phospho y1054 + y1059 antibody (1:50, Abcam ab5473) in RBB and 2.5% donkey serum overnight at 4°C. The retina was then washed with 1:1 RBB:PBS and bound antibodies were visualized using Dylight 488-conjugated streptavidin (1:100, Vector Laboratories) and Alexa Fluor-546 conjugated donkey anti-rabbit IgG (1:100, Life Technologies) in RBB + 2.5% donkey serum for 2 hours. The retina was sliced into four flat quadrants, transferred to positively charged glass slides, and mounted with Fluoromount-G (SouthernBiotech 0100-01) prior to coverslipping and sealing.
4.2.7 Thick-Section Immunostaining of Mouse Skeletal Muscle

For thick-section immunostaining of skeletal muscle, mice were sacrificed by isoflurane overdose and perfused sequentially with PBS and 4% PFA via left ventricle cannulation at physiologic pressure. EDL muscles were dissected, immersed in 4% PFA for 2 hours, cryoprotected with 15% sucrose for 2 hours and 30% sucrose overnight at 4°C. Tissues were then embedded in OCT embedding medium (Tissue-Tek) and stored at -80°C. 100-µm thick longitudinal cryosections were permeabilized with 0.5% Triton-X 100 in PBS and double immunostained using biotinylated rat anti-mouse CD31 antibody (1:25, BD Biosciences 553371) and rabbit polyclonal anti-VEGF receptor 2 phospho y1054 + y1059 antibody (1:50, Abcam ab5473) or rabbit polyclonal anti-NG2 antibody (1:200, Millipore AB 5320). Bound antibodies were visualized using Dylight 488-conjugated streptavidin (1:100, Vector Laboratories) and Alexa Fluor-546 conjugated donkey anti-rabbit IgG (1:100 or 1:200, Life Technologies). Nuclei were visualized with TO-PRO-3 iodide (1:500, Life Technologies). Thick-sections were transferred to positively charged glass slides, mounted with Fluoromount-G (SouthernBiotech 0100-01) and flanked with 100-µm thick plastic coverslip spacers (Thermo Scientific) prior to coverslipping and sealing.

4.2.8 Laser Scanning Confocal Microscopy and 3-Dimensional Reconstruction

Mouse retina endothelial tip cells, and uninjured capillaries and mother vessels in EDL muscle were imaged with an LSM 510 Meta Confocal Microscope (Zeiss) using a 40x water-immersion objective and Argon2 (488 nm excitation), HeNe1 (543 nm excitation), and HeNe2 (633 nm excitation) lasers, generating up to 50, 1 µm-thick z-slices at 2048x2048 image resolution. Z-slices were reconstructed into 3-dimensional maximum intensity projections with Image Viewer and Zen (Zeiss) software. To determine the localization of VEGFR2 activation within mother vessels and intraluminal protrusions or pillars, 3-dimensional reconstructions were scrutinized through each of their individual optical sections and orthogonal XZ and YZ projections in order to identify locations of pVEGFR2 signal. pVEGFR2 signal was considered to be lateralized on intraluminal protrusions and pillars if the signal was localized and enriched on only
one side of the pillar, with the dividing axis perpendicular to the direction of flow. pVEGFR2 signal was deemed to be non-lateralized if signal was localized on both sides of the pillar with similar enrichment.

HUVEC monolayers on glass coverslips were transferred facedown with the center exposed onto flanking 100-µm thick plastic spacers (Thermo Scientific), then placed atop positively charged glass slides covered with Prolong Gold Antifade Mountant (Thermo Scientific). HUVECs were imaged with an LSM 510 Meta Confocal Microscope (Zeiss) using a 40x water-immersion objective and Argon2 (488 nm excitation), and HeNe1 (543 nm excitation) lasers, generating up to 40, 0.5 µm-thick z-slices at 1024x1024 image resolution. Z-slices were reconstructed into 3-dimensional maximum intensity and surface projections with Zen (Zeiss) software. To quantify the surface area (µm²) of HUVECs, individual cells were manually circumscribed using ImageJ. To quantify the thickness (µm) of HUVECs orthogonal XZ and YZ projections were generated with Zen (Zeiss) software, and the maximum thickness of individual cells was measured manually using ImageJ.

4.2.9 Analysis of Red Blood Cell Velocity and Vessel Wall Shear Rate

Red blood cell velocities ($V_{RBC}$ [µm/sec]) in individual microvessels were quantified from video files using space-time images as described previously (Ellis et al., 1992, Japee et al., 2005, Arpino et al., 2017). Briefly, 1-second (21 frames) interval mean red blood cell velocities were calculated using custom MATLAB software, generating a 2D gray scale plot of RBC location change with time. This enabled an unbiased quantification of RBC transit velocities within microvessels throughout the entire EDL muscle. Mean $V_{RBC}$ averaged over a 15-second imaging time-frame within each microvessel was determined in a total of 115 uninjured capillaries, 82 microvessels 5 days following surgery, and 93 microvessels 7 days following surgery. Experimentally derived vessel wall shear rate [sec⁻¹] was calculated using the equation shown below as described by Al-Khazraji et al., with vessel lumen diameter (D) and maximum velocity (vessel centerline velocity, $V_{Expt Max}$) used as equation inputs (Al-Khazraji et al., 2016). Vessel wall shear rate was calculated in 48 microvessels five days following surgery.
ranging from 7.3 – 38.5 μm in diameter.

\[
\text{Vessel Wall Shear Rate} = \frac{\left(\frac{44x(D) + 1.99}{166x(D) + 0.39}\right) \times V_{\text{Expt Max}}}{0.022x(D) + 0.00074}
\]

4.2.10 Semi-Thin Sectioning

Semi-thin sectioning of hindlimb anterior muscles was performed as described for the mouse renal corpuscle (Roth et al., 2015). Mice were sacrificed by isofluorane overdose, and perfused sequentially with PBS and 4% PFA via left ventricle cannulation at physiologic pressure. Anterior hindlimb muscles were dissected and immersed in Karnovsky’s fixative (3% Glutaraldehyde, 2% formaldehyde in 0.1 M Sorenson buffer, Electron Microscopy Sciences, 15731-10) overnight at 4°C. Tissues were then subjected to a second round of fixation the next day in fresh Karnovsky’s fixative for one hour and dehydrated in increasing concentrations of electron microscopy-grade ethanol solutions (Electron Microscopy Sciences) in ddH₂O as follows: 50% for 1.5 hours, 70% for 1 hour, 95% for 1 hour, and 100% for 1 hour. Next, the tissue was infiltrated with 100% propylene oxide for 1 hour (Electron Microscopy Sciences) and then infiltrated with a 1:1 mixture of propylene oxide and embedding medium (5 mL Embed-812, 3mL Araldite, 11 mL DDSA, and 0.3 mL DMP-30, Electron Microscopy Sciences) for 1 hour. Next, the tissue was infiltrated overnight with 100% embedding medium at room temperature. The next day, tissue was subjected to fresh 100% embedding medium for 1 hour. All aforementioned fixation, dehydration, propylene oxide infiltration, and embedding medium infiltration steps were performed within glass vials continuously spun on an orbital rotator. Tissues were placed in pre-constructed wells (Electron Microscopy Sciences) along with 100% embedding medium using a syringe with a blunt-tip 18½ gauge needle and incubated for 72 hours at 60°C in order to polymerize the medium.

For sectioning, tissue blocks were trimmed with steel razor blades into a truncated pyramid with a trapezoid cut block face, with the upper and lower edges being parallel. The adhesive Pattex Compact (Henkel KGaA, Dusseldorf, Germany) was diluted in xylene (6 drops of xylene for a 5 mm³ volume of adhesive) and applied to the underside
of the block with a needle tip for generating tissue section ribbons. Glass slides used to mount sections were treated prior to sectioning to remove their water-retention properties by immersion in an ethanol (96%) ammonia (25%) solution at a ratio of 9:1 for at least 1 day and then scrubbed with a sponge and dish soap and thoroughly rinsed with distilled water. Tissue blocks were sectioned into semi-thin (0.5 µm thickness) serial ribbons using a Histo Jumbo diamond knife (Diatome AG, Biel, Switzerland) and ultramicrotome (Reichert- Jung Ultracut E). Once ribbons were generated, treated glass slides were carefully slid into the Histo Jumbo diamond knife water trough and the floating ribbons were gently guided onto the slide. ddH₂O was added to the slide and placed on a hotplate for 1 hour at 60°C.

For staining of semi-thin sections, polychromatic and methylene blue stains were prepared according to Sato et al. and D’Amico (Sato et al., 1973, D’Amico, 2005). Polychromatic stain was prepared by dissolving monobasic sodium phosphate (0.5 g, Sigma), basic fuchsine (0.25 g, Electron Microscopy Sciences), and methylene blue (0.2 g, Electron Microscopy Sciences) in 15 mL of 0.5% boric acid solution, followed by the addition of 70 mL of ddH₂O and 10 mL of 0.72% NaOH (pH 6.8). Methylene blue stain was prepared by dissolving methylene blue (0.75 g, Electron Microscopy Sciences), azure B (0.25 g, Electron Microscopy Sciences), and borax (0.5 g, Electron Microscopy Sciences) in 100 mL of ddH₂O. Approximately 1 mL of polychromatic solution was added to the slides placed on a hot plate at 55°C for 5 minutes, rinsed with ddH₂O, and then followed by the addition of 1 mL of methylene blue stain for 10 seconds. Serial sections were mounted with Eukitt mounting medium (Sigma, 03989) and coverslipped. Up to 1000 serial sections were obtained from a single tissue block, and 3 native EDL muscles and 3 EDL muscles harvested five days following surgery were sectioned. Stained serial sections were digitally scanned using a ScanScope CS brightfield digital slide scanner with a 40x objective (Aperio Technologies, Vista, CA, USA). Digital micrographs of serial sections were captured using Sedeen Viewer Software and exported as TIFF image files. Up to 50 serial TIFF image files of individual microvessels were rotated and aligned semi-automatically in Photoshop (Adobe). Videos were generated using 3D Slicer (Fedorov et al., 2012).
4.2.11 In-Vivo Blockade of VEGFR2 Signaling

For acute in-vivo blockade of VEGFR2 signaling, mice were injected intraperitoneally with individual VEGFR2-selective chemical tyrosine kinase inhibitors or an anti-VEGFR2 antibody 4.5 days following surgery. Specific details for each individual inhibition regimen are described in detail below. To determine the effect of acute VEGFR2 signaling inhibition on microvascular network architecture, blue-light epifluorescence intravital microscopy of the EDL muscle was performed on day 5 following surgery (16-18 hours after injection), as above.

*Apatinib (YN968D1):* Apatabin (Selleckchem, S2221) is a selective VEGFR2 tyrosine kinase inhibitor with an IC50 of 1 nM. A working solution was prepared by dissolving 10mg of Apatinib powder into sterile 560 µL dimethyl sulfoxide (DMSO, Sigma) and 560 µL modified PBS (140 mM NaCl, 9 mM of Na₂HPO₄, 1.3 mM of NaH₂PO₄, pH 7.4) with continuous agitation. On day 4.5 following surgery, 168 µL of Apatinib solution (60 mg/kg) was injected intraperitoneally into mice (Tong et al., 2012, Tian et al., 2011, Mi et al., 2010, Lin et al., 2014). An equivalent volume of sterile vehicle solution was administered intraperitoneally into littermate controls. IVVM was performed 16-18 hours following administration.

*Cabozantinib and ZM 323881:* Cabozantinib (Selleckchem, XL184, BMS-907351) and ZM323881 HCl (Selleckchem, S2896) are selective VEGFR2 tyrosine kinase inhibitors with an IC50 of 0.035 nM and <2 nM, respectively. Working solutions were prepared by dissolving Cabozantinib or ZM323881 HCl into sterile 2% DMSO (Sigma), 30% polyethylene glycol 300 (PEG-300, Sigma), 5% Tween-80 (Sigma), and 63% ddH₂O, according to the manufacturers instructions. On day 4.5 following surgery, 375 µL of Cabozantinib solution (30 mg/kg) or 400 µL of ZM323881 HCl solution (60 mg/kg) was injected intraperitoneally into mice (Zhao et al., 2016, Sameni et al., 2016, Phan et al., 2015). Sterile vehicle solution was administered intraperitoneally into littermate controls. IVVM was performed 16-18 hours following injection.

*DC101:* DC101 (BioXCell, BE0060) is a monoclonal anti-mouse in-vivo VEGFR2 blocking antibody. On day 4.5 following surgery, 200 µL of DC101 in sterile
PBS pH 7.0 (40 mg/kg) or 200 µL of isotype control (BioXCell, BE0088, 40 mg/kg) was injected intraperitoneally into mice (Ding et al., 2015, Arulanandam et al., 2015, Larrayoz et al., 2014). IVVM was performed 16-18 hours following administration.

4.2.12 Statistics

Statistical analyses were performed using Prism 5 (Graphpad Software). All values passing D’Agostino and Pearson omnibus normality test are presented as mean ± SEM. Those not passing the normality test are presented as median and IQR. Comparisons among normally distributed variables were made by t-test or analysis of variance with Bonferonni’s post hoc test. Comparisons among non-normally distributed variables were made by Mann-Whitney test or Kruskall-Wallis test with Dunn’s post hoc test. Linear regression analyses were used to assess relationships between vessel wall shear rate and diameter. Statistical significance was set at P<0.05.

4.3 Results

4.3.1 Vascular Regeneration in Ischemic Muscle Entails the Emergence of Large Caliber Primordial Vessels

To study the processes by which a microvascular network regenerates following ischemic injury to skeletal muscle, C57Bl/6 mice were subjected to right femoral artery excision. The extensor digitorum longus (EDL) muscle and microvasculature was then evaluated daily for five days by histology. Hematoxylin and eosin staining revealed that the entire EDL muscle was infarcted on day one following surgery (Figure 4.1 a,c). This was evident through palely stained myocytes with absent nuclei (Figure 4.1 c). A similar appearance was seen on days two and three. On day four an inflammatory response emerged (Figure 4.1 e). On day five there was extensive inflammation with clearing of necrotic muscle debris (Figure 4.1 g). Myocyte regeneration initiated on day five, as evidenced by the appearance of miniature hyper-eosinophilic satellite cells with central nuclei in the spaces where the muscle was destroyed (Figure 4.1 g).
Figure 4.1 Histology of the EDL muscle following ischemic injury and necrosis and the emergence of primordial vessels

Hematoxylin and eosin-stained sections of EDL muscle before surgery (A), one day following surgery showing palely stained myocytes with absent nuclei (C), four days following surgery showing the emergence of an inflammatory response (arrows, E), and five days following surgery showing the appearance of satellite cells with central nuclei in the spaces where the muscle was destroyed (arrows, G).

Fluorescence micrographs of EDL muscle immunostained for CD31 (green) showing capillaries (arrows) surrounding myofibers (asterisk) before surgery (B), near complete loss of capillaries after ischemic insult one day following surgery (D), the absence of capillaries four days following surgery (F), and the emergence of a neovasculature comprised of dilated primordial vessels (arrows) with thin CD31+ walls five days following surgery (H). Nuclei are visualized with DAPI.
Native

Day 1

Day 4

Day 5

Hematoxylin and Eosin

CD31 Immunofluorescence

Native

Day 1

Day 4

Day 5

Hematoxylin and Eosin

CD31 Immunofluorescence
Immunostaining for CD31 revealed near-complete loss of CD31-stained capillaries by day one and complete absence by day 3, consistent with my previous findings (Arpino et al., 2017) (Figure 4.1 b,d). Microvessels remained absent on day four (Figure 4.1 f). Remarkably however, on day five, there was a seemingly abrupt appearance of new vascular structures that stained positively for the EC marker CD31 (Figure 4.1 h). These primordial neovessels were much larger than normal capillaries, up to 31 µm in diameter, but the walls of the neovessels were thin (Figure 4.1 b,d).

4.3.2 Primordial Vessels in Regenerating Skeletal Muscle Lack Microvascular Network Hierarchy or Specification

To evaluate primordial vessels at a network level, I performed daily real-time microscopy of the EDL surface zone following intravenous injection of FITC-labeled Dextran (Arpino et al., 2017). High-magnification RBC transit maps of native EDL muscle revealed a highly organized microvascular network containing parallel capillaries (Figure 4.2 a). RBC transit was completely absent on days one through four following surgery (Figure 4.2 b,c). On day five, RBC transit maps revealed the large caliber, primordial vessels as the first flow-receiving channels in the EDL muscle following femoral artery excision (Figure 4.2 d). Networks of primordial vessels were first detected in the distal-most region of the EDL surface.

Quantitative analysis highlighted the striking dilatation of primordial vessels compared to normal capillaries (12.2 (9.6 – 17.0) vs 5.5 (5.1 – 6.0) µm, median (IQR), P<0.0001) (Figure 4.2 e). Moreover, individual primordial vessels exhibited remarkable intravessel diameter variability along the vessel segment length, in contrast to normal capillaries (Figure 4.2 a,d,f). Furthermore, the primordial network architecture had no evidence for hierarchy or specification, in contrast to the hierarchical arteriole-capillary-venule (ACV) units of normal microvascular networks (Figure 4.3).
Figure 4.2 RBC transit maps of primordial vessels

A. RBC transit map of native EDL muscle microvasculature showing a highly organized network with capillaries (arrow) arranged in parallel between individual muscle fibers.

B-C. RBC transit maps of EDL muscle on one (B) and four (C) days after femoral artery excision showing no RBC transit after ischemic injury.

D. RBC transit map of EDL muscle five days after femoral artery excision showing the emergence of a flowing neo-microvasculature comprised of primordial vessels (arrows).

E. Graph showing box and whisker (10-90 percentile) plot of microvessel lumen diameter as evaluated from 164 capillary segments in 3 native EDL muscles and from 377 neovessels in 5 EDL muscles on day five following surgery. *p<0.0001.

F. Line plot depiction of intravessel diameter heterogeneity along the vessel segment length in a representative normal capillary (n=1) and a representative primordial vessel (n=1).
Day 4

Day 5

Lumen Diameter (µm)

Primordial Vessel

Native Capillary

Length Along Vessel Segment (Quintiles)
Figure 4.3 Network architecture of primordial vessels

High-magnification RBC transit maps of the native EDL muscle microvasculature (left) and the primordial neo-microvasculature that emerges five days following femoral artery excision (right). Networks of primordial vessels (right, arrows) bear little resemblance to native capillaries or hierarchical arteriole-capillary-venule units in native EDL muscle (left, A=arteriole, C=capillary, V=venule).
4.3.3 Primordial Vessels are Mother Vessels that Rapidly Transform Into an Extensive Microvascular Network

In Chapter 3, I established that an extensive microvasculature with arteriole-capillary-venule hierarchy regenerates in the infarcted and regenerating EDL muscle (Arpino et al., 2017). Remarkably, immunostaining for CD31 revealed that by day seven dilated primordial channels were no longer detectable. Instead, I found a microvascular network with lumens of smaller caliber. These vessels were widely dispersed throughout the tissue and at the interface of newly formed muscle fibers (Figure 4.4 a,b). RBC transit maps confirmed that the primordial channels had rapidly transformed into a widely distributed and arborized microvascular network within 48 hours, including the emergence of neocapillaries (Figure 4.4 c-e).

Quantitative analysis of RBC transit maps revealed that the lumen diameter of neo-microvessels nearly halved by day seven (6.6 (5.1 – 8.8) µm D7 vs 12.2 (9.6 – 17.0) µm D5, P<0.0001, Figure 4.4 f), and were only slightly enlarged compared to normal capillaries in native EDL muscle (6.6 [5.1 – 8.8] D7 vs 5.5 [5.1 – 6.0] µm native, P<0.0001, Figure 4.4 f). Moreover, the 48-hour network transformation entailed a 1.5-fold increase in the number of vessel bifurcations (P=0.002, Figure 4.4 g) and a concomitant tripling of vascular density (P<0.0001, Figure 4.4 h). Together, these findings reveal that the dilated primordial vessels were, in fact, “mother vessels” (MVs) that rapidly transformed into an extensive microvascular network.

4.3.4 The Mother Vessel Wall is Comprised of Collectivized Endothelial Cells and NG2+ Pericytes

To characterize the cellular composition of the MV I double-immunolabeled 100-µm thick sections of EDL muscle for the EC marker, CD31, and the mural cell marker, NG2. I then generated 3-dimensional reconstructions. This revealed that MVs were comprised of collectivized CD31+ ECs (Figure 4.5 b). However, in contrast to native EDL muscle capillaries that are comprised of single elongated ECs positioned in series along the vessel length, the circumference of MVs at a given site was typically comprised
Figure 4.4 Transformation of mother vessels into an extensive microvasculature over 48 hours

**A.** Hematoxylin and eosin-stained section of EDL muscle seven days following surgery showing newly formed muscle fibers with central nuclei (arrows).

**B.** Fluorescence micrograph of EDL muscle immunostained for CD31 (green) on day seven following surgery showing smaller caliber microvessels (arrows) surrounding newly formed muscle fibers (asterisk). Nuclei are visualized with DAPI.

**C-E.** RBC transit maps of EDL muscle on days five (C), six (D), and seven (E) following surgery showing the transformation of mother vessels into a widely distributed microvasculature over 48 hours. Hatched lines denote EDL muscle borders.

**F.** Graph depicting box and whisker (10-90 percentile) plot of microvessel lumen diameter, evaluated as indicated below the growth (n=3-5 EDL muscles for each time point). *p<0.0001.

**G.** Graph depicting branching density of EDL muscle microvascular networks (n=3-5 EDL muscles for each time point). *p=0.001, **p=0.002.

**H.** Graph depicting vascular density of EDL muscle microvascular networks (n=3-5 EDL muscles for each time point). *p<0.0001.
Figure 4.5 Mother vessel wall structure and composition

**A-B.** Confocal micrographs of projected optical sections of a native EDL muscle capillary (A) and a mother vessel on day five following surgery (B) immunolabeled for CD31 (green) and TO-PRO-3 (blue).

**C. Left:** Higher magnification confocal micrograph of projected optical sections of a mother vessel on day five following surgery. Shown are two EC nuclei (arrows, hatched lines denote edges of nuclei) that comprise the circumference of the MV at the same location along the vessel segment length. **Right:** Fluorescence micrographs of native and day five EDL muscle cross-sections immunostained for CD31 (green) showing a single EC nucleus in a normal capillary (top, arrow) and three EC nuclei in a mother vessel (bottom, arrows).

**D.** Confocal micrographs of projected optical sections of the mother vessel shown in Figure 4.5 b immunolabeled for NG2 (red) and TO-PRO-3 (blue). On the right, the CD31 (green) channel is overlaid.
of more than one EC (Figure 4.5 a-c). Interestingly, MVs were also extensively invested with NG2+ pericytes, whereas native capillaries were only sparsely invested with pericytes that did not fully wrap the circumference of the vessel (Figure 4.5 d). Thus, MVs are a structurally unique conduit in skeletal muscle.

### 4.3.5 Mother Vessels Have Profoundly Slow Blood Flow and Low Wall Shear Rate

I next evaluated the RBC transit dynamics within MVs. To do this, I performed real-time microscopy to image RBCs in transit over seven days following surgery. This revealed that, on day five, RBCs within MVs transited with a velocity ($V_{RBC}$) of 87.6 (54.1-177.4) µm/s (median (IQR)) (Figure 4.6 a). This was only 17% of that in native EDL muscle capillaries ($P<0.0001$, Figure 4.6 a). Moreover, MVs had strikingly high hematocrit, with up to 7 RBCs identified across the vessel segment diameter (Figure 4.6 c). This was in contrast to the single-file RBC transit that is characteristic of native capillaries (Fig. 4.6 b). Furthermore, although MV lumens contained an abundance of RBCs and were of similar caliber to skeletal muscle feeder arterioles, RBC transit dynamics were non-arteriolar as they did not exhibit the parabolic radial $V_{RBC}$ profile that is characteristic of skeletal muscle arterioles (Al-Khazraji et al., 2016). Instead, the radial $V_{RBC}$ profile was blunted and venous-like, with the vessel centerline $V_{RBC}$ being similar to $V_{RBC}$ at the vessel wall (data not shown).

I also assessed RBC transit dynamics during the transformation of MVs into an arborized microvasculature. RBC transit velocity nearly doubled to 164.9 (121.0-236.1) µm/s by day seven following the transformation of network architecture ($P<0.0001$, Figure 4.6 a). Moreover, fewer RBCs were identified across vessel segment lumens on day seven as a consequence of reduced diameter. Single-file RBC transit was identified in capillary-like neovessels, although this was not widespread at this time. As well, RBC transit remained slow compared to the native EDL microvasculature ($P<0.0001$, Figure 4.6 a).
Figure 4.6 Mother vessel RBC transit dynamics

A. Box and whisker (10-90) percentile plot of red blood cell velocities ($V_{RBC}$) in individual microvessels within native and regenerated EDL muscle evaluated on the designated days following surgery. Values were obtained from a total of 307 microvessels. Each value is derived from RBC velocities, averaged over a 15-second imaging time frame, within each microvessel. *p<0.0001, **p=0.004, †p=0.002.

B. Video still frame depicting single-file RBC transit in native capillaries. Hatched lines denote capillary lumens.

C. Video still frames depicting non-single file RBC transit in MVs with high hematocrit. Hatched lines denote MV lumens.
A

Red Blood Cell Velocity (µm/s)

Native  Day 4  Day 5  Day 6  Day 7

B

Native

Day 5

Day 5
I next evaluated vessel wall shear rate using a recently published strategy (Al-Khazraji et al., 2016). MV wall shear rate within 48 MVs with a mean diameter of 19.6 ± 8 µm was calculated to be 89.5 (26.4-161.8) sec\(^{-1}\) (median (IQR)) and ranged from 7.1 to 606.4 sec\(^{-1}\) (Figure 4.7). This is ~11-fold lower than the calculated wall shear rate in a typical native capillary (1028 sec\(^{-1}\)). Moreover, there was an inverse relationship between the diameter of MVs and wall shear rate (\(r^2 = 0.177, P=0.003, \) Figure 4.7). These findings reveal profoundly low shear forces on the ECs of MVs.

### 4.3.6 Mother Vessels Contain Micro-Obstructions to RBC Transit

I next asked how MVs transformed into an arborized neo-microvasculature. To study this, I inspected RBC transit maps of MVs at high resolution on days five and six for evidence of active microvascular growth. Surprisingly, I discovered that there were micro-obstructions to RBC transit within the flowing MV lumens. Micro-obstructions revealed themselves along the length of MV segments and near MV bifurcations as punctate dots or circular entities, 2-8 µm in diameter, that were devoid of RBC transit signal (Figure 4.8). Complementary real-time microscopy video sequences confirmed that “dead-zones” were bona fide micro-regions of no flow. Individual RBCs were seen to deflect in transit around the dead zones, indicating that they were intraluminal micro-obstructions. In addition, cell-free blood plasma “streams” of ~5 µm in length were observed immediately downstream of the micro-obstructions. Consistent with this, the RBC transit map “dead-zones” appeared larger than the actual micro-obstruction identified in the video sequence, indicating there was a region downstream of the micro-obstruction where no RBCs were flowing.

In addition, I also identified MV micro-divisions that were larger than micro-obstructions. Micro-divisions were identified in RBC transit maps as ellipsoid “dead-zone” entities deficient of any RBC motion (Figure 4.9). Micro-divisions were 3-10 µm in width and 5-50 µm in length. Real-time microscopy video sequences confirmed that these regions were devoid of blood flow. Moreover, I identified MVs wherein the lumen had effectively divided and “split” the long axis of the vessel (Figure 4.10). The MV
Figure 4.7 Mother vessel wall shear rate

Scatter plot with regression line depicting inverse relationship between mother vessel wall shear rate and lumen diameter on day five following surgery. (n=48 microvessels from 5 EDL muscles on day 5 following surgery, \( r^2=0.177 \), \(*p=0.0002\)). Data points represent individual vessels.
The scatter plot shows the relationship between lumen diameter (in micrometers, µm) and vessel wall shear rate (in seconds^{-1}). The data points are spread across the x-axis, which represents the lumen diameter, and the y-axis, which represents the vessel wall shear rate. A linear regression line is fitted to the data, with the equation \( r^2 = 0.177 \) and a significance level of \( p=0.003 \). This indicates a moderate negative correlation between the two variables.
**Figure 4.8 RBC transit maps of micro-obstructions to RBC transit**

RBC transit maps of circular “dead-zone” entities (micro-obstructions, arrows) devoid of RBC transit signal within flowing MV lumens. Images are from 5 EDL muscles on day 5 following surgery. Multiple images are presented to depict the variety of sizes and shapes of micro-obstructions, the location of micro-obstructions within either straight regions of MVs or at branch points, and the presence of both isolated and adjacent micro-obstructions. To note, micro-obstructions were often identified at a bulbous site of locally expanded MV diameter.
Figure 4.9 RBC transit maps of mother vessel micro-divisions

RBC transit maps showing ellipsoid “dead-zone” entities (micro-divisions, arrows) devoid of RBC transit signal within the flowing mother vessel lumen. Images are from 5 EDL muscles on day 5 following surgery. Multiple images are presented to show that micro-divisions can be isolated or adjacent to one or more similar divisions. To note, like punctate micro-obstructions, micro-divisions tend to be at an expanded site of the primitive vessel.
Figure 4.10 RBC transit maps of mother vessel splits

A. RBC transit maps showing mother vessel splits (arrows), wherein RBCs are diverted into two daughter vessel segments that are spread apart from one another and reconverge into a single mother vessel lumen downstream.

B. RBC transit map showing a mother vessel that has asymmetrically split (arrow) into a capillary-caliber daughter vessel (arrowhead) and a residual mother vessel.

C. RBC transit maps showing multiple obstructions to RBC transit in series along the length of individual mother vessels (arrows).
splits were larger than the identified micro-divisions and up to 200 µm in length. This entailed the diversion of RBCs into two distinct and smaller daughter vessel segments that were spread apart from one another, and a downstream reconvergence of the RBCs into a single MV lumen and flow path (Figure 4.10 a). Remarkably, MVs were also found to split into daughter vessels of capillary caliber (Figure 4.10 b). Furthermore, multiple micro-obstructions, micro-divisions, and vessel splits were found positioned in series along the length of individual MVs (Fig 4.10 c). Collectively, these findings point to a series of events that is initiated by a micro-obstruction that grows and rapidly doubles vessel content through splitting.

4.3.7 Micro-Obstructions to RBC Transit are Identified in Mother Vessels with Ultra-Low Shear

I next determined if the presence of a micro-obstruction or micro-division within a MV lumen was associated with the size of the vessel. To evaluate this, I quantified the maximum lumen diameter at the site of local MV expansion that contained these specific entities. This revealed that, at the site of micro-obstructions and micro-divisions, MVs had exceptionally large lumen diameters (micro-obstructions: 24.7 ± 1.8 µm, micro-divisions: 21.4±2.1 µm, Figure 4.11 a). Interestingly, the maximum lumen diameter of daughter vessels between sites of MV divergence and reconvergence was found to be 47% narrower than that of MVs at the site of micro-obstructions (P=0.0001, Figure 4.11a) and 39% narrower than that of MVs at the site of micro-divisions (P=0.007, Figure 4.11 a).

In addition, I determined if the presence of a micro-obstruction or micro-division with a MV lumen was associated with specific RBC transit dynamics. To evaluate this, I quantified the vessel wall shear rate at the site of local MV expansion that specifically contained these entities. This revealed that at the site of micro-obstructions, MVs exhibited ultra-low vessel wall shear rates (46.4 (23.9-120.4) sec\(^{-1}\), Figure 4.11 b). Interestingly, the vessel wall shear rate in daughter vessels was 3-fold higher than that in MVs at the site of micro-obstructions (P=0.027, Figure 4.11 b). Furthermore, micro-
Figure 4.11 Lumen diameter and wall shear rate of mother vessels at the site of micro-obstructions and micro-divisions

A. Graph depicting the lumen diameter of mother vessels containing micro-obstructions, mother vessels containing micro-divisions, and daughter vessels on either side of a mother vessel split. Values were obtained from a total of 47 vessels in 5 EDL muscles on days five or six following surgery (n=17 micro-obstructions, n=16 micro-divisions, n=14 daughter vessels). *p=0.0001, **p=0.007.

B. Box and whisker plot (10-90 percentile) of the wall shear rate in mother vessels containing micro-obstructions, mother vessels containing micro-divisions, and daughter vessels on either side of a mother vessel split. Values were obtained from a total of 47 vessels in 5 EDL muscles on days five or six following surgery (n=17 micro-obstructions, n=16 micro-division, n=14 daughter vessels). *p=0.027

C. RBC flux heat-map showing the identification of a micro-obstruction (arrow) located in a large mother vessel with low blood flow, the identification of a micro-division in a large mother vessel with low blood flow (arrowhead), and the absence of a RBC transit micro-obstruction in the smaller mother vessel with faster flow on the left (asterisk).
obstructions or micro-divisions to RBC transit were not identified within daughter vessels.

As shown in the RBC flux heat-map in Figure 4.11c, micro-obstructions and micro-divisions were identified in large caliber MV sites with low flow. Moreover, no micro-obstructions or micro-divisions were identified in relatively smaller microvessels with faster flow. Collectively, these results indicate that micro-obstructions and micro-divisions are harbored within sites of MVs that have particularly enlarged lumens and ultra-low shear forces.

**4.3.8 Intraluminal Micro-Obstruction is a Protruding Endothelial Cell**

To identify a structural correlate to the RBC transit-free micro-zones, I cross-sectioned through EDL muscle five days following surgery. Hematoxylin and eosin staining of 5-µm thick cross-sections revealed the presence of translumenal obstacles within dilated, thin-walled channels (Figure 4.12 a,b). This is consistent with intraluminal tissue “pillars” within the flowing MV.

To determine the cellular structure of intraluminal pillars, I performed serial semi-thin tissue sectioning. This entailed generating tissue ribbons of up to 1000, 0.5-µm thick serial sections from a single tissue block of EDL muscle (Figures 4.13, 4.14, 4.15). Next, I scrutinized MV sections on a section-by-section basis to identify intraluminal tissue pillars. This revealed ECs protruding into the MV lumen space (Figure 4.13, 4.14, 4.15).

Two types of EC pillars were identified: 1) thin EC cytoplasmic finger-like protrusions (Figure 4.13 images 7+12, Figure 4.14 image 9) and 2) thicker protrusions comprised of the EC body and nucleus (Figure 4.13 image 19, Figure 4.15 image 17). Protrusions could be seen to emanate from opposing sides of the vessel wall (Figure 4.13, images 17-19). Translumenal connections could extend along the vessel length as little as 2 µm (Figure 4.14 images 9-13). Furthermore, there could be more than one protrusion at the same cross-sectional site within a MV (Figure 4.13, Figure 4.15 image 19). I also observed that the nucleus of the pillar-forming EC was typically enlarged and rounded,
Figure 4.12 Histology of translumenal obstacles within mother vessels

A-B. Hematoxylin and eosin-stained 5-µm cross-sections of EDL muscle showing translumenal obstacles to RBC transit within MV lumens (arrows).
Figure 4.13 Serial semi-thin sections of intraluminal EC protrusions (ribbon 1)

Photomicrographs of serial semi-thin (0.5-μm) sections that show two distinct EC intraluminal protrusions (arrows). Serial images are numbered sequentially. In images 7 and 12, a thin EC cytoplasmic finger-like protrusion is indicated. In image 19, a thicker protrusion comprised of the EC body and nucleus is indicated (arrow). In image 17, a protrusion that emanates from both opposing sides of the vessel is indicated (arrows). RBCs can be seen within the MV lumen.
Figure 4.14 Serial semi-thin sections of intraluminal EC protrusions (ribbon 2)

Photomicrographs of serial semi-thin (0.5-µm) sections depicting an EC intraluminal protrusion (arrows). Serial images are numbered sequentially. Images 1-20 show a thin EC cytoplasmic protrusion that spans the lumen of the MV (arrows). Two sectioned RBCs can also be seen within the lumen.
Figure 4.15 Serial semi-thin sections of intraluminal EC protrusions (ribbon 3)

Photomicrographs of serial semi-thin (0.5-µm) sections depicting multiple EC intraluminal protrusions (arrows). Serial images are numbered sequentially. Images 1-20 show a thin EC cytoplasmic protrusion that spans the lumen of the MV. RBCs can be seen within the lumen. In image 17, a thicker protrusion comprised of an EC body and nucleus is indicated (arrow). In image 19, two EC protrusions are indicated at the same site within the MV (arrows). RBCs can be seen within the lumen.
instead of the flat and elongated nucleus characteristic of ECs aligned to the direction of blood flow (Levesque et al., 1985) (Figure 4.12 a, Figure 4.14).

**4.3.9 Pillar Cells Express CD31, Endomucin, and VE-Cadherin**

To confirm and characterize the EC identity of the pillars, I immunostained the EDL muscle on day five using a panel of distinct EC markers. This revealed that the intraluminal pillars stained positively for CD31, endomucin, and VE-Cadherin (Figure 4.16 a-c). Interestingly, immunoreactivity for CD31 and VE-Cadherin within the pillar appeared more intense than the surrounding MV wall endothelium. As well, CD31 and VE-Cadherin were broadly co-localized (Figure 4.16 c). I did not identify filopodia on the protrusions by immunostaining for endomucin, which labels the filopodia on sprouting endothelial tip cells (Gianni-Barrera et al., 2013) (Figure 4.16 b).

**4.3.10 Endothelial Cell Intraluminal Protrusions Have Low and Lateralized VEGFR2 Activation**

VEGF-VEGFR2 signaling is fundamental to sprouting angiogenesis. In addition, endothelial tip cell selection is based on relatively heightened VEGFR2 activation (Gerhardt et al., 2003). Thus, I sought to probe for VEGFR2 expression and activity in EC pillars. To do this, I performed immunostaining and generated 3-dimensional confocal reconstructions. Specifically, I double-immunostained for an EC marker and phosphorylated VEGFR2 (pVEGFR2) at tyrosine residues 1054 and 1059. Autophosphorylation of these tyrosine residues, located in the intracellular tyrosine kinase activation loop, is necessary for VEGFR2 signaling and maximal kinase activity (Claesson-Welsh, 2003, Kendall et al., 1999, Simons, 2012, Dougher et al., 1999, Lanahan et al., 2010). Moreover, phosphorylation of these residues is required for VEGF-induced sprouting angiogenesis (Kawamura et al., 2008).

I first immunostained the neonatal mouse retina on P5 for isolectin-B4 and pVEGFR2 as a positive control. Generation of 3-dimensional reconstructions of the developing retina microvasculature revealed a focal enrichment of pVEGFR2 at endothelial sprouting tips and on cell filopodia (Figure 4.17). In contrast, pVEGFR2 was
Figure 4.16 Endothelial cell identity of intraluminal protrusions

A-C. Photomicrographs of mother vessels and translumenal endothelial pillars (arrows) immunostained for CD31 (A,C), endomucin (B), and VE-Cadherin (C), as indicated by the respective arrows. 5-µm thick immunostained sections were imaged by brightfield or widefield fluorescence microscopy. Nuclei are visualized with DAPI.
Figure 4.17 VEGFR2 activation in mouse retina sprouting tip cells

Confocal micrographs of projected optical sections of sprouting endothelial tip cells within the developing mouse retina on P5 immunolabeled for isolectin-b4 (green) and pVEGFR2 y1054+1059 (red). Hatched lines denote sprouting tip and arrows point to focal enrichment of pVEGFR2 at sprout tip. Non-endothelial background pVEGFR2 signal likely is VEGFR2 activity in retinal microglia, astrocytes, and resident macrophages (Gerhardt et al., 2003)
reduced at sites distant to the tip.

Double immunostaining for an EC marker and pVEGFR2 in EDL muscle 5-µm thick cross-sections, as well as 100-µm thick longitudinal sections, revealed no pVEGFR2 signal in native capillaries. (Figure 4.18 a,c). In contrast, the MV wall endothelium exhibited widespread and intense immunoreactivity for pVEGFR2. This was apparent both within EC cytoplasm and at EC-EC junctions (Figure 4.18 a,b,d,e). Some non-endothelial interstitial cells within the complex EDL microenvironment on day five were intensely positive for pVEGFR2 immunoreactivity (Figure 4.18 b).

In 3-dimensional confocal reconstructions of the long axis of MVs, ECs protrusions and pillars appeared as CD31-intense circular structures within the lumen (Figure 4.18 e, arrowhead). Remarkably, although widespread pVEGFR2 signal was evident within the MV wall EC population, optical sectioning through the intraluminal EC protrusions and pillars revealed low pVEGFR2 signal in these structures (Figure 4.19 a-c). Interestingly, intraluminal protrusions and pillars were not entirely devoid of VEGFR2 activation and highly focal pVEGFR2 signal could be identified (Figure 4.19 a,b, arrowheads). This finding is arguably paradoxical because sprouting angiogenesis exhibits the reverse pattern - the protruding tip is high in pVEGFR2 signal.

I also determined that 93% of EC protrusions and 83% of translumenal EC pillars had focal pVEGFR2 signal lateralized to one side of the structure (Figure 4.20 b-e). The remainder had pVEGFR2 signal localized on both sides of the translumenal structure. Lateralized VEGFR2 activation may have been induced by ligand-independent VEGFR2 phosphorylation by shear stress, given that there is no matrix-bound VEGF gradient in the blood. Regardless, these results indicate that intraluminal EC protrusion initiation is associated with differential VEGFR2 signaling among MV ECs, including reduced and localized VEGFR2 activation in protrusions and pillars.
Figure 4.18 Widespread VEGFR2 activation in mother vessel wall endothelium

A-B. Photomicrographs of native and day five injured EDL muscle double-immunostained for endomucin (green) and pVEGFR2 1054+1059 (red). Nuclei are visualized with DAPI. Arrows point to native capillaries with no pVEGFR2 signal (A, left) and mother vessels with widespread intense, immunoreactivity for pVEGFR2 (A right, B). Asterisk in B indicates EDL interstitial cells positive for pVEGFR2 immunoreactivity.

C. Confocal micrograph of projected optical sections of a native EDL muscle capillary immunostained for CD31 (green), pVEGFR2 1054+1059 (red), and TO-PRO-3 (blue).

D-E. Confocal micrographs of projected optical sections of mother vessels immunostained for CD31 (green), pVEGFR2 1054+1059 (red), and TO-PRO-3 (blue). Widespread pVEGFR2 immunoreactivity is shown in the MV wall endothelium (arrows) and a CD31-intense circular-appearing structure consistent with an intraluminal protrusion is shown in E (arrowhead).
Figure 4.19 Reduced and highly localized VEGFR2 activation in EC protrusions and pillars

A. Confocal orthogonal XZ-axis optical projections of MVs showing CD31-high pVEGFR2-low intraluminal EC protrusions, relative to surrounding MV wall endothelium. Arrows indicate intraluminal protrusions, arrowheads indicate focal pVEGFR2 signal.

B. Confocal orthogonal YZ-axis optical projections of MVs showing CD31-high pVEGFR2-low intraluminal EC protrusions relative to surrounding MV wall endothelium. Arrows indicate intraluminal protrusions, arrowheads indicate focal pVEGFR2 signal.

C. Confocal z-axis optical sections of MVs showing CD31-high pVEGFR2-low intraluminal EC protrusions relative to surrounding MV wall endothelium. Arrows indicate intraluminal protrusions.
Figure 4.20 Lateralized pVEGFR2 signal on intraluminal protrusions

A. 3-dimensional schematic of a mother vessel containing an intraluminal EC pillar (blue = nucleus, green = pillar cytoplasm, red = lateralized pVEGFR2) showing the specific location of confocal z-sections presented in (B).

B. Confocal z-axis optical sections of a mother vessel containing an intraluminal EC pillar with pVEGFR2 signal lateralized to one side of the translumenal pillar (arrows).

C. Confocal orthogonal XZ-axis optical projections of the mother vessel presented in (B) showing the face of the translumenal pillar with lateralized pVEGFR2 signal (arrow).

D. Confocal z-axis optical section of a mother vessel containing an intraluminal EC pillar with pVEGFR2 signal lateralized to one side of the “hollow” translumenal pillar (arrow). Pillar is outlined by hatched line.

E. Pie charts showing the proportion of intraluminal protrusions (n=14), and translumenal pillars (n=21) exhibiting lateralized pVEGFR2 signal.
Lateralized pVEGFR2
Non-Lateralized pVEGFR2

Translumenal Pillars
Intraluminal Protrusions

7% 93%
17% 83%

Lateralized pVEGFR2
Non-Lateralized pVEGFR2
4.3.11 Intussusceptive Angiogenesis Increases Upon Inhibition of VEGFR2 Signaling In Vivo

Because I identified that intraluminal protrusions were associated with reduced pVEGFR2 signal, I next asked if blockade of VEGFR2 signaling in MVs would impact IA. To study this, I injected mice intraperitoneally with a VEGFR2-selective tyrosine kinase chemical inhibitor on day 4.5 following surgery and assessed the microvasculature by immunostaining and real-time microscopy 16 hours later (5 days after femoral artery excision). Immunostaining revealed that injection of the VEGFR2-selective tyrosine kinase inhibitor, Apatinib (60 mg/kg), on day 4.5 resulted in reduced pVEGFR2 immunoreactivity throughout the EDL muscle and neo-vasculature on day 5 (Figure 4.21 a). However, the formation of MVs was not abolished (Figure 4.21 a). Real-time microscopy of the EDL muscle on day five revealed that Apatinib induced a modest reduction in EDL surface zone vascularity (P=0.002, Figure 4.21 c), but the resulting network was similar in vascular and branching density compared to that of mice injected with vehicle control (Figure 4.21 d,e).

However, analysis of RBC transit maps revealed that Apatinib delivery resulted in distinctive MV crowding and increased branching complexity of MVs (Figure 4.21 b). Remarkably, I also identified increased pillar content and increased vessel splitting density (Figure 4.21 f,g). Apatinib induced a 2.6-fold increase in the network density of intraluminal pillars (P=0.0003, Figure 4.21 f) and a 2.5-fold increase in the network density of vessel splits (P<0.0001, Figure 4.21 g), relative to that of mice injected with vehicle.

To ascertain if the network-level increase in IA was a result of VEGFR2 signaling blockade versus off-target effects of Apatinib, I repeated the experiment using either a more potent VEGFR2 tyrosine kinase inhibitor (Cabozantinib) or a more selective VEGFR2 tyrosine kinase inhibitor (ZM323881 HCl) (Yakes et al., 2011, Whittles et al., 2002). Analysis of RBC transit maps revealed that short-term delivery of Cabozantinib or ZM323881 HCl induced a 2.2-fold and 2.7-fold increase in the network density of pillars, respectively (P=0.018, P=0.005, Figure 4.22 a-c) and 1.7-fold and 2.0-fold increase in
Figure 4.21 Histology and RBC transit maps of mother vessels following inhibition of VEGFR2 signaling by Apatinib delivery

A. Photomicrographs of 5-µm thick EDL muscle sections on day five immunostained for endomucin (green) and pVEGFR2 y1054+1059 (red) following intraperitoneal injection of Apatinib or vehicle control on day 4.5. Nuclei are visualized with DAPI.

B. RBC transit maps of the EDL muscle neovasculature on day five following intraperitoneal injection of Apatinib or vehicle control on day 4.5. Arrows point to sites of mother vessel intraluminal pillars or splits.

C-E. Graphs depicting total microvasculature length (C), vascular density (D), and branching density of EDL muscle microvascular networks. (n=5 vehicle control EDL muscles, n=3 Apatinib EDL muscles) (*p=0.002).

F-G. Graphs depicting the numerical density of pillars (F) and splits (G) normalized to total microvasculature length. (n=5 vehicle control EDL muscles, n=3 Apatinib EDL muscles) (*p=0.0003, **p<0.0001).
Apatinib

**Materials and Methods**

To investigate the effect of Apatinib on endothelial cell proliferation and migration, we performed a series of experiments on a murine model of vascular remodeling. The primary focus was to evaluate the impact of Apatinib on the network density of pillars, the network density of splits, the vascular density, and the total network length.

**Results**

**A**

Day 5 + Vehicle

Day 5 + Apatinib

**B**

Vehicle (DMSO/PBS)

Apatinib

**C**

Total Network Length (mm) / EDL Muscle Surface Zone

**D**

Vascular Density (µm / µm² vascularized zone)

**E**

Branch Density (mm network length)

**F**

Network Density of Pillars (# / mm vessel length)

**G**

Network Density of Splits (# / mm vessel length)

* and ** indicate statistical significance.
**Figure 4.22 RBC transit maps of mother vessels following inhibition of VEGFR2 signaling by Cabozantinib, ZM323881 HCl, and DC101 delivery**

**A-B.** RBC transit maps of the EDL muscle neovasculature on day five following intraperitoneal injection of Cabozantinib (A), ZM323881 HCl (B), or vehicle control on day 4.5. Arrows point to sites of mother vessel intraluminal pillars or splits.

**C-D.** Graphs depicting the numerical density of pillars (C) and splits (D) normalized to total microvasculature length. (n=3 vehicle control EDL muscles, n=3 Cabozantinib EDL muscles, n=2 ZM323881 HCl EDL muscles) (*p=0.0003, **p<0.0001) (†p=0.017).

**E.** RBC transit maps of the EDL muscle neovasculature on day five following intraperitoneal injection of DC101 or HRPN antibody control on day 4.5. Arrows point to sites of mother vessel intraluminal pillars or splits.

**F-G.** Graphs depicting the numerical density of pillars (C) and splits (D) normalized to total microvasculature length. (n=2 DC101 EDL muscles, n=2 HRPN EDL muscles) (*p=0.0003, **p<0.0001) (†p=0.017).
A

Vehicle (DMSO/PEG-300/Tween-80/ddH2O)

Cabozantinib

100 µm

ZM323881 HCl

Network Density of Pillars
(#/mm vessel length)

Network Density of Splits
(#/mm vessel length)

C

HRPN

DC101

Network Density of Pillars
(#/mm vessel length)

Network Density of Splits
(#/mm vessel length)

D

E

HRPN

DC101

F

G

0.6

0.4

0.2

0.0

0.6

0.4

0.2

0.0

0.15

0.10

0.05

0.00

HRPN

DC101

HRPN

DC101

Vehicle

Cabozantinib

ZM323881 HCl

F

G

0.6

0.4

0.2

0.0

0.15

0.10

0.05

0.00

HRPN

DC101

Vehicle

Cabozantinib

Vehicle

Cabozantinib

ZM323881 HCl

ZM323881 HCl
the network density of vessel splits, respectively (P=0.017, Figure 4.22 a,b,d), relative to that of mice injected with vehicle.

I also blocked VEGF-VEGFR2 signaling by delivering the anti-VEGFR2 antibody DC101 (Fontanella et al., 2014). In contrast to inhibition of VEGFR2 signaling by tyrosine kinase chemical inhibitors, delivery of DC101 induced only a modest and non-significant increase in the network density of pillars, relative to that of mice injected with IgG HRPN antibody control (Figure 4.22 e,f). Moreover, the network density of vessel splits was not different following DC101 delivery (Figure 4.22 g).

Therefore, blockade of intracellular VEGFR2 signaling with selective tyrosine kinase chemical inhibitors resulted in increased pillar formation and intussusceptive angiogenesis.

**4.3.12 Downregulation of VEGFR2 Expression Induces Endothelial Cell Rounding, Decreases Cell Spreading, and Enables EC Protrusive Behaviour in EC Monolayer In Vitro**

The foregoing studies suggested that relatively low VEGFR2 signaling enabled pillar formation and IA. In this context, it is noteworthy that depletion of VEGFR2 in culture has been found to inhibit the alignment of HUVECs to the direction of laminar flow and shear stress (Coon et al., 2015, van der Meer et al., 2010). Thus, I next tested if differential EC VEGFR2 expression impacted EC morphology within an EC monolayer. For this, I co-cultured HUVECs with baseline VEGFR2 expression and reduced VEGFR2 expression, at a 10:1 ratio. The HUVECs were transfected before co-culture with either control siRNA or VEGFR2 siRNA, and also transduced with RFP or GFP adenovirus, respectively. VEGFR2 mRNA knockdown in HUVECs was confirmed by qRT-PCR (Figure 4.23 a). Next, I examined the co-cultured monolayers by laser scanning confocal microscopy and generated 3-dimensional reconstructions of optical sections. This revealed that ECs with VEGFR2 knockdown were less spread and had a smaller planar surface area (Figure 4.23 b,c).
Figure 4.23 Reduction of cell spreading following targeted VEGFR2 reduction

A. Graph depicting relative mRNA abundance of VEGFR2 in HUVEC cultures transfected with control (n=3) or VEGFR2 (n=3) siRNA (*p=0.005).

B. Box and whisker plot (10-90 percentile) depicting the surface area of HUVECs transfected with control (n=174) or VEGFR2 (n=34) siRNA (*p=0.006).

C. Confocal micrographs of projected optical sections of HUVEC monolayers transfected with control (red) or VEGFR2 (green) siRNA showing reduced cell spreading and surface area in HUVECS with reduced VEGFR2 expression.
A

HUVEC VEGFR2 Abundance (ΔCT)

Control siRNA
VEGFR2 siRNA

B

HUVEC Surface Area (µm²)

Control siRNA
VEGFR2 siRNA

C

Control siRNA
VEGFR2 siRNA

Control siRNA
VEGFR2 siRNA

Control siRNA
VEGFR2 siRNA
Figure 4.24  Cell rounding following targeted VEGFR2 reduction

A. Box and whisker plot (10-90 percentile) depicting the maximum cell body thickness of HUVECS transfected with control siRNA (n=149) or VEGFR2 siRNA (n=36) (*p<0.0001). Control ECs were transduced with RFP and VEGFR2-knockdown ECS with GFP.

B. Orthogonal confocal projections of HUVEC monolayers transfected with control siRNA (red) or VEGFR2 siRNA (green) showing cell bulging and increased thickness of HUVECS with reduced VEGFR2 expression (arrows).

C. Orthogonal confocal projections of HUVEC monolayers, with reversed fluorophore adenoviral transduction, and transfected with control siRNA (green) or VEGFR2 siRNA (red) showing cell bulging and increased thickness of HUVECS with reduced VEGFR2 expression (arrows).
To search for protrusive EC behaviour, I evaluated orthogonal optical sections of the monolayers. This revealed that VEGFR2 knockdown induced cell rounding and thickening in the z-axis (Figure 4.24 a,b). The VEGFR2-knockdown HUVECs were often positioned at a different vertical plane relative to the surrounding monolayer. To confirm that the increased thickening was not imaging artifact introduced by GFP, I reversed the adenovirus fluorescent labeling. This revealed that RFP-labeled VEGFR2 knockdown HUVECs were also rounder and thicker in the z-axis. (Figure 4.24 c). To further assess for protrusive capacity in VEGFR2-low ECs, I treated the monolayer with sphingosine-1-phosphate (S1P) (1 µM, 3 minutes). This exacerbated the morphologic heterogeneity of co-cultured HUVECs, and induced striking EC protrusions (Figure 4.25 a). Lamellipodial protrusions in VEGFR2-low ECs formed and were seen to spread on the surface of adjacent ECs. This contrasted with the normal contact-inhibited behaviour of VEGFR2-normal ECs in a monolayer (Figure 4.25 a). As well, the S1P-stimulated VEGFR2-knockdown cells were small (P=0.0006, Figure 4.25 a,c), rounded (P=0.007, Figure 4.25 b,d), and bulged in the vertical direction. Thus, the overall appearance of VEGFR2-low ECs was that of “rogue” cells that are capable of protruding out of the EC collective.

4.4 Discussion

Using high-resolution real-time imaging of RBCs in transit, I have identified architectural and cellular events by which a new microvascular network forms following ischemic injury in skeletal muscle. These events are: 1) the abrupt emergence of metastable primordial microvessels that are subjected to ultra-low blood flow and shear forces, and 2) the rapid transformation of these MVs into an extensive and hierarchical microvasculature. This later event proceeds by pillar formation and vessel spitting and is enabled by heterogeneous activation of VEGFR2.

Intussusceptive Angiogenesis in Ischemic Skeletal Muscle

Studies have attributed the neovascularization response in ischemic skeletal muscle to sprouting angiogenesis (Hershey et al., 2001, Madeddu, 2005, Limbourg et al., 2009, Al Haj Zen et al., 2010). Sprouting endothelial tip cells are known to generate a
Figure 4.25 Non-contact inhibited cellular protrusions in VEGFR2-low ECs induced by S1P

A-B. Confocal micrographs of projected (A) and orthogonal (B) optical sections of HUVEC monolayers transfected with control siRNA (red) or VEGFR2 siRNA (green) and treated with 1 µM S1P for 3 minutes showing bizarre cellular protrusions (A, arrows) and exaggerated cell thickening (B, arrow).

C. Graph depicting the surface area of HUVECs transfected with control (n=93) or VEGFR2 (n=17) siRNA and treated with 1 µM S1P for 3 minutes (*p=0.0006).

D. Graph depicting the maximum cell body thickness of HUVECs transfected with control (n=65) or VEGFR2 (n=15) siRNA and treated with 1 µM S1P for 3 minutes (*p=0.007).
Control siRNA + S1P

VEGFR2 siRNA + S1P

HUVEC Thickness (µm)

HUVEC Surface Area (µm²)

A

B

YZ - Ortho View

C

D

Control siRNA + S1P

VEGFR2 siRNA + S1P

Control siRNA + S1P

VEGFR2 siRNA + S1P

*
web-like meshwork of microvessels and capillaries (Gerhardt et al., 2003). However, the parallel and long-running (200-500 µm in length) capillaries in skeletal muscle bear little similarity to this pattern. Interestingly, the EDL microvasculature regenerated over 48 hours, whereas it takes eight to ten days to generate a complete microvasculature in the mouse retina via sprouting (Gerhardt et al., 2003). I have demonstrated that the neomicrocirculation in skeletal muscle is reconstructed following ischemic injury via the formation of transluminal pillars - the hallmark of IA, not sprouting angiogenesis.

Intussusceptive, or splitting, angiogenesis is less understood than sprouting angiogenesis. Only recently is IA becoming recognized as a more general mechanism of blood vessel growth during development and postnatal growth (De Spiegelaere et al., 2012). This is the first study to have identified IA during neovascularization in ischemic and regenerating skeletal muscle. My findings do not exclude concurrent sprouting angiogenesis although, at least in muscle zones where the native microvasculature is obliterated, IA appears dominant. Immunostaining for CD31 and endomucin, the latter of which homogeneously stains ECs including the filopodia on sprouting tip cells (Gianni-Barrera et al., 2013), did not reveal endothelial tip cells emanating from the MV wall. Moreover, semi-thin sectioning showed no evidence for the degradation of MV basement membrane, which is a requirement for sprouting angiogenesis (Carmeliet et al., 2011). As well, filopodia were not identified on the “inward” EC protrusions. Together with real-time microscopy data, these results indicate that the transformation of MVs into an extensive microvasculature occurred by splitting angiogenesis and not by sprouting.

**The Mother Vessel**

My discovery of primordial and slow-flowing channels within the obliterated skeletal muscle was surprising. I termed these dilated channels MVs as they are reminiscent of the transient, very enlarged, and thin-walled “mother vessels” found as the first new vessels that form in tumors (Chang et al., 2009). A potentially similar structure has been noted in skin and muscle in pre-existing microvessels that dilate in response to substantial local VEGF delivery (Pettersson et al., 2000). However, in the regenerating EDL muscle MVs were de-novo vascular structures, as we have shown that the pre-
existing microvasculature is lost following femoral artery excision (Arpino et al., 2017). Thus, MVs in infarcted and regenerating skeletal muscle are previously undescribed vascular structures that generate daughter vessels by IA.

The rapidity with which the MV transformed into an extensive microvasculature was remarkable. This entailed the formation of translumenal EC pillars and subsequent division of MVs into smaller conduits. This process doubled the local vascular density and generated bifurcations. This organ-wide reconstruction of a neo-microvasculature over 48 hours reveals the efficiency of pillar formation and IA for regenerative angiogenesis. The result was the prompt restoration of vascular and branching density in the EDL seven days following its obliteration.

**Mother Vessel Blood Flow and Pillar Formation**

The wide caliber of MVs and slow RBC transit suggest profoundly non-physiological perfusion. Furthermore, real-time microscopy revealed that EC pillars were located in MVs with particularly large diameters and particularly low blood flow. Interestingly in the chick chorioallantoic membrane (CAM), pillars have also been found to form in vessels with widened lumens or circumference irregularities (Lee et al., 2010). These findings raise the possibility that perturbed shear forces may be related to pillar formation. My findings support this. Quantitative analysis established that MVs had ultra-low vessel wall shear rates. The average wall shear rate in MVs was only 4% compared to that of small arterioles (21 µm diameter) in the rat gluteus maximus muscle (Al-Khazraji et al., 2016) and 5% of that of arterioles in rat cremaster muscle (Koller et al., 1991). In fact, MV wall shear rates were lower than that of a healthy venous circulation, where the lowest shear rates in the microcirculation are normally found. For example, shear rates have been reported to be ~400 sec\(^{-1}\) in cat mesentery venules (25 µm diameter) (Lipowsky et al., 1978) and ~600 sec\(^{-1}\) in rat mesentery venules (Pearson et al., 2000). I also observed RBC aggregations (rouleaux), a phenomenon seen under conditions of low blood flow shear (Shiga et al., 1983).

I propose that the low MV wall shear rate was permissive for pillar development. This is consistent with computational simulations of the CAM vasculature (Lee et al.,
2010) and calculation of low shear rates (100 sec\(^{-1}\)) in tumor vessels undergoing IA (Kamoun et al., 2010). Moreover, my finding pillars in series is supported by a computational model which predicted that new pillars would form downstream of pre-existing pillars, at sites of low shear stress (Lee et al., 2010). This suggests that shear rate gradients guide the insertion and growth of pillars. The process of MV splitting may also serve to increase and normalize vessel wall shear rate.

Paradoxically, the low blood flow and low shear forces that I found during IA contrasts with what others have observed associated with IA in healthy skeletal muscle. Zhou et al. and Milkiewicz et al. demonstrated that delivering prazosin to rats increased the shear forces within native EDL muscle capillaries and that this intraluminal stimulus induced IA (Zhou et al., 1998a, Milkiewicz et al., 2001, Egginton et al., 2001). Reconciling high shear- versus low shear-induced IA warrants further study.

**Molecular Regulation of Intussusceptive Angiogenesis: VEGFR2 Signaling**

Using an antibody targeting phosphorylated VEGFR2 intracellular tyrosine residues 1054 and 1059, residues critical for the maximal kinase activity of VEGFR2 (Dougher et al., 1999), I identified low and highly localized VEGFR2 activation in the EC comprising an intraluminal protrusion or pillar. In contrast, there was extensive VEGFR2 activation in the ECs comprising the rest of the lining of the MV wall. This finding is opposite that found during sprouting angiogenesis. For sprouting angiogenesis, the VEGF-VEGFR2 signaling axis is the master controller of tip cell formation and guidance (Olsson et al., 2006, Gerhardt et al., 2003). ECs with the greatest activation of VEGFR2 become a sprouting tip cell (Gerhardt et al., 2003, Jakobsson et al., 2010). Therefore although from a structural standpoint, IA appears as an “outside-in” form of sprouting angiogenesis, the signaling events appear to be regulated quite differently.

Both VEGF (Dougher and Terman, 1999, Simons, 2012, Harper and Bates, 2008, Dellinger and Brekken, 2011) and blood flow shear forces (Jin et al., 2003, Tzima et al., 2005, Chen et al., 1999) can induce the phosphorylation of VEGFR2 1054 and 1059. My finding of lateralized pVEGFR2 signal on one side of translumenal EC pillars raises the possibility of flow-related, ligand-independent activation of the receptor. ECs detect
blood flow shear forces via an EC-specific mechanosensory complex. This mechanosensory complex is comprised of CD31, VE-Cadherin, and VEGFR2 (Tzima et al., 2005). This complex is required for the alignment of ECs in the direction of blood flow and entails phosphorylation of VEGFR2 at tyrosine residue 1054 (Tzima et al., 2005). Although I do not know whether the activated VEGFR2 was on the upstream or downstream side of the pillar, it is reasonable to expect the former. In fact, this is supported by computational simulations (Filipovic et al., 2009). I also cannot rule out the possibility that circulating VEGF is binding to and phosphorylating VEGFR2 on the pillar.

I did not study the signaling pathways downstream of VEGFR2 activation that may be implicated in pillar formation. It is likely that EC cytoskeletal rearrangements and migratory behaviour are required to generate an intraluminal protrusion. In this regard, phosphorylation of VEGFR2 intracellular tyrosine residues 951, 1175, and 1214 are implicated in EC cytoskeletal and shape control and warrant further study in IA (Coon et al., 2015, Koch et al., 2011, Holmqvist et al., 2004, Olsson et al., 2006, Sun et al., 2012).

**Augmentation of IA During Regenerative Angiogenesis by Inhibiting VEGFR2 Activity**

Acute blockade of intracellular VEGFR2 signaling using three different selective tyrosine kinase chemical inhibitors, remarkably, increased network-level pillar formation and IA. This suggests that low VEGFR2 signaling, in itself, is a driver of pillar formation and IA. The in vivo blocking findings are consistent with a study of gene expression in the developing porcine glomerulus, which demonstrated that VEGFR2 is downregulated during late glomerular development where IA predominates over sprouting angiogenesis (De Spiegelaere et al., 2010). Moreover, another study demonstrated that during glomerular repair following injury, IA was not halted following delivery of the VEGFR2 and PDGFRβ tyrosine kinase inhibitor PTK787/ZK222584 (Wnuk et al., 2011).

Interestingly, blocking VEGF-induced VEGFR2 dimerization and activation via acute delivery of DC101 did not significantly impact the formation of pillars and IA. In contrast to the chemical inhibitors, DC101 binds the extracellular domain of VEGFR2 (Fontanella et al., 2014). Whether this extracellular-targeted VEGFR2 blockade strategy
could also block ligand-independent activation of VEGFR2 is not completely understood. However, assuming it does not, my results support the importance of ligand-independent activation of VEGFR2 in MVs and the heterogeneity of this response in pillar formation and IA.

I did not observe a substantial reduction in the overall vascularity of the regenerated EDL muscle surface zone following VEGFR2 blockade. This may be explained by the fact that I delivered only a single dose of VEGFR2 inhibitor and evaluated the network ~16 hours later. This is in contrast to the reduction of vascularity observed in tumors following delivery of VEGFR2 inhibitors over several weeks (Ackermann et al., 2012, Tong et al., 2004, Goel et al., 2011, Chung et al., 2010).

**Protrusive Behaviour of VEGFR2-Low Endothelial Cells**

ECs normally elongate in response to physiological shear stress, with their long axis oriented in the direction of flow (Dewey et al., 1981, Krueger et al., 1971). Importantly, VEGFR2 knockdown has been shown to decrease the alignment of ECs in the direction of laminar shear stress (Coon et al., 2015, van der Meer et al., 2010). I propose that those pVEGFR2-low ECs in MVs are relatively insensitive to shear stress-induced EC alignment, priming these particular ECs to protrude into the MV lumen. This was supported by my in vitro model of VEGFR2 knockdown. This demonstrated that HUVECs with reduced VEGFR2 expression were less spread and thicker relative to their control cell neighbours. VEGFR2 knockdown also induced the overlapping of cell bodies and the sending of cytoplasmic projections over or under neighbouring control cells. Accordingly, I speculate that reduction of VEGFR2 signaling activity induced amoeboid, non-contact inhibitable protrusive behaviour in ECs.

These “amoeboid-like” phenomena were exaggerated when the VEGFR2 knockdown HUVEC actin cytoskeleton was acutely triggered to reorganize by delivery of S1P. S1P is a soluble sphingolipid that normally stimulates EC spreading and migration and stabilizes the cortical actin network (Vouret-Craviari et al., 2002, Yamaoka-Tojo et al., 2004). S1P is also a negative regulator of sprouting angiogenesis and inhibits VEGF-VEGFR2 signaling (Gaengel et al., 2012). Interestingly, epithelial cell
extrusion in response to overcrowding requires the S1P receptor 2 pathway (Gu et al., 2011). Together, these findings further suggest that pillar-forming ECs are a phenotypic subset of ECs in the MV that are relatively undifferentiated and prone to extrusion.

Finally, I propose that pillar-forming ECs in the regenerating skeletal muscle exhibit “flow-seeking” behaviour. Positioning a cellular protrusion or pillar into the flowing lumen might be a strategy for an EC to increase the shear forces that it encounters, by making more contact with flowing RBCs. This flow-seeking cellular strategy might be particularly important if the EC had an underdeveloped VEGFR2 activation machinery. Moreover, this might serve to help differentiate the pillar-forming EC into a more mature microvascular EC phenotype.

In summary, early-stage vascular regeneration following skeletal muscle infarction entails the emergence of metastable, primordial microvessels with ultra-low blood flow. These primordial microvessels subsequently transform into a neo-microvascular network via inward EC protrusion and IA. This non-sprouting angiogenesis cascade was determined by differential VEGFR2 activation in ECs.

4.5 References


CHAPTER 5

General Discussion and Conclusions

The findings presented in this thesis provide several new insights into the microvasculature that forms upon angiogenesis in adult ischemic tissues. As a methodological centerpiece, I developed an intravital microscopy-based strategy for interrogating the architecture, hierarchy, perfusion attributes, and vasoreactivity of the newly formed microcirculation in adult mouse tissues. I then used this strategy to uncover five new phenomena. First, I identified multiple microcirculatory defects within malignant renal tumors. Second, I discovered that FGF9 can productively reconfigure and “normalize” the architecture and otherwise abnormal hierarchy of the tumor microvasculature and also impart vasoreactivity. Third, I discovered a range of defects in the regenerated microvasculature that forms in skeletal muscle following ischemic injury. These architectural and functional abnormalities established that the microcirculation was ill-suited for effective O₂ delivery. Fourth, I uncovered a non-sprouting form of angiogenesis by which the early microvascular network forms following skeletal muscle infarction. This entailed inward endothelial cell protrusion and intussusceptive angiogenesis. Finally, I discovered that intussusceptive angiogenesis in regenerating skeletal muscle was based on differential VEGFR2 activation.

5.1 Development of a Novel Strategy for Interrogating Microvascular Architecture and Perfusion in Tissues

The approach I developed for interrogating newly formed microvessels entailed real-time epifluorescence intravital microscopy. I was thus able to image RBCs in transit and ascertain flow-based assessments of the microcirculation. This included quantifying RBC velocity and flux in individual microvessels and interrogating microvascular vasoreactivity. The latter was enabled by developing chamber devices that were integrated into the microscope stage for locally delivering vasoactive agents, or perturbing local O₂ content in the tissues while simultaneously tracking RBC motion. Together, these methodological developments advanced the field by enabling an
assessment of the regenerated microvasculature by integrating network microstructure with real-time tracking of RBC transit.

5.2 Novel Insights into the Structure, Function, and “Normalization” of Renal Tumor Microcirculation

Tumors recruit a vascular network to sustain their growth. The resulting vessels are generally understood to be tortuous with abnormally large diameters (Nagy et al., 2009, Fukumura et al., 2010). My findings add several new insights into the renal tumor vascular biology. RBC transit analysis revealed not only densely packed and tortuous microvessels, but abnormalities in network hierarchy and vasoreactivity. I found that network hierarchy was essentially missing and that flow appeared venous-like throughout. I also found that vasoreactivity was minimal. These abnormalities constitute important reasons for impaired O\textsubscript{2} delivery in the tumor.

I also discovered that delivery of FGF9 productively normalizes the renal tumor microcirculation. This intervention generated hierarchical arteriole-capillary-venule microcirculatory units and feeder vessels with vasomotor competence. Together, these normalized RBC transit and improved blood flow within the renal tumor. The attainment of a physiologically advanced microvasculature with the ability to dynamically regulate blood flow is, to my knowledge, new in the field of tumor biology.

These findings have implications for tumor therapy. The perfusion anomalies arising from an absence of network hierarchy and vasomotor control would impair the delivery of chemotherapeutic drugs. Likewise, the associated hypoxia is likely to blunt the response to radiation therapy (Jain, 2005, De Bock et al., 2011). In this regard, FGF9-mediated normalization of the tumor microvascular network architecture and function has the potential to enhance the delivery of chemotherapeutics and improve the effectiveness of radiation therapy.

5.3 Future Directions in Tumor Angiogenesis and Vascular Normalization Studies

Whether the FGF9-exposed tumor can regulate blood flow in response to local O\textsubscript{2} content remains unknown. I found that FGF-exposed tumors had functional \( \alpha \)-adrenergic
and nitric oxide signaling. Establishing whether the feeder vessels can also regulate flow in response to a local hypoxia “challenge” would clarify the idea that FGF9 delivery can normalize microvascular responsiveness, with implications for increasing tumor sensitivity to radiation therapy (De Bock et al., 2011, Goel et al., 2011).

I studied the FGF9-exposed renal tumor 10 days following implantation of Renca cells. However, a daily microscopy-based investigation via an abdominal window chamber could reveal powerful insights into the processes of tumor angiogenesis and FGF9-induced normalization (Ritsma et al., 2013). A window chamber strategy might, for example, elucidate the relative contributions of sprouting versus intussusceptive angiogenesis in the generation of the renal tumor microvasculature (Patan et al., 1996, Nico et al., 2010).

The current paradigm of tumor vessel normalization is to balance the chronic pro-angiogenesis drive in tumors with anti-angiogenesis and vascular destroying drugs (Goel et al., 2011). Importantly, I showed that delivery of a growth factor (FGF9) normalizes the vasculature and blood flow in tumors and also avoids vascular destruction. These findings could be translatable to a clinical setting. First, however, whether normalization of network hierarchy and restoration of vasoreactivity augments delivery of chemotherapeutic drugs should be directly tested in vivo. One could test if the “normalized” network effectively distributes chemotherapeutics throughout a large tumor volume and leads to tumor shrinkage. A similar pre-clinical study of radiation could also be employed.

5.4 The Regenerated Skeletal Muscle Microvasculature is Profoundly Flawed and Ill-Suited for Oxygen Delivery

I determined the physiological attributes of the regenerated microcirculation that forms in the mouse hindlimb EDL muscle following femoral artery excision. I discovered that, despite extensive regeneration of the previously obliterated microvasculature, the neo-microvasculature was highly flawed. I found abnormalities in network-level architecture, supernumerary branching, violations of arteriole-capillary-venule geometry and hierarchy, slow and monotonous RBC transit, a blunted hyperemic response to
hypoxia, and aberrant smooth muscle cell wrapping architecture. Moreover, these flawed attributes were largely permanent. Associated with this, hypoxia persisted in the regenerated skeletal muscle (Figure 5.1). These newly identified pathologies would be effectively hidden using current strategies for assessing microvascular perfusion (Limbourg et al., 2009, Simons et al., 2015, Arpino et al., 2017).

Notably, this is the first study to assess regenerative angiogenesis in ischemic skeletal muscle at the microcirculatory network level and at the individual capillary level where gas and nutrient exchange occurs. The remarkable discordance between the microvascular restoration process and the functionality of the O₂ delivery system indicates that capillary density and bulk flow are inadequate indicators of functional angiogenesis. Accordingly, I propose that strategies beyond stimulating angiogenesis in ischemic muscle are required, including normalizing microcirculatory physiology.

5.5 Future Directions in Regenerative Angiogenesis Studies

Following femoral artery excision in the C57Bl/6 mouse strain, the entire EDL muscle was destroyed and all subsequently identified microvessels were new vascular structures. However, this raises the consideration as to how does the nature of the injury determine the functional outcome of the angiogenic stimulus? Little is known about the angiogenesis process in muscle following minor ischemia, i.e. without extensive tissue infarction. Likewise, the response to traumatic injury or chemical damage is not well understood. Importantly, the limb damage can be more restricted and focal in these latter settings and upstream blood flow is not halted. Thus, the resulting networks might be comprised of a mix of pre-existing and new microvessels. It is also not known if the natural angiogenesis program in response to exercise produces a healthier and more functional network compared to a regenerated network in muscle (Egginton et al., 2001, Olfert et al., 2010). These questions could be addressed using the high-resolution strategies employed in this thesis.

In the big picture, my studies have identified targets within the microvascular regeneration program that must be improved or “normalized” in order to advance therapeutic strategies for treating ischemic disease in patients. Some of the identified
Figure 5.1 The regenerated microcirculation in ischemic skeletal muscle is flawed and ineffective at delivering oxygen

Schematic of the network and cellular architecture of the proximal (feeder) end of microcirculatory units in normal skeletal muscle microcirculation (top) and regenerated skeletal muscle microcirculation (bottom). In the healthy skeletal muscle microcirculation (top), arteries and arterioles are tightly invested with circumferentially wrapped SMCs and SMC processes. In the regenerated skeletal muscle microcirculation (bottom), there are substantial gaps in coverage between individual SMCs and SMC processes (arrows). As well, some SMC processes are not oriented perpendicularly to the axis of the underlying endothelial channel (arrowhead). These wrapping abnormalities, together with downstream arterio-venous shunts and hyper-dense capillary beds, impair appropriate delivery of O$_2$ and produce a hypoxic tissue environment.
defects, such as the aberrant SMC wrapping, represent a component of the faulty neo-microvasculature that could be modulatable. Co-delivery of the growth factor FGF9 with pro-angiogenesis growth factors has the potential to improve and reinforce the wrapping of neo-terminal arterioles with SMCs (Frontini et al., 2011, Yin et al., 2015, Said et al., 2013). This would be expected to impart the reconstructed microvasculature with improved vasomotor control and better delivery of RBCs and O₂ in response to demand. The development of drug or growth factor delivery platforms for individuals with peripheral vascular disease thus remains a potentially important therapeutic context for this study.

Furthermore, the development of minimally invasive and high-resolution imaging strategies to be used in patient tissues could substantially enhance the understanding of angiogenesis and microcirculatory pathophysiology in the clinical setting. As such technology is developed, my findings could serve as an important framework for identifying microvascular health versus pathology.

5.6. Discovery of a Non-Sprouting Form of Angiogenesis Following Skeletal Muscle Infarction

I discovered that early-stage vascular regeneration following skeletal muscle infarction entails the emergence of primordial, large-caliber vessels that subsequently transform into a neo-microvascular network. This transformation proceeds via intraluminal EC protrusion and IA. This is the first study to have identified the process of IA during neovascularization in ischemic skeletal muscle. Sprouting angiogenesis was not identified during this early-stage process even though the neovascularization response in ischemic skeletal muscle has been attributed to sprouting angiogenesis (Hershey et al., 2001, Madeddu, 2005, Limbourg et al., 2009, Al Haj Zen et al., 2010). Moreover, current therapeutic angiogenesis strategies for ischemic muscle target pathways controlling sprouting angiogenesis (Annex, 2013, Said et al., 2013). However, therapeutically driven neovascularization in the ischemic skeletal muscle has been ineffective at improving perfusion (Annex, 2013, Simons et al., 2003). My findings provide novel information in this regard and prompt the reconsideration of current angiogenesis paradigms and
associated therapeutic strategies for ischemic disease.

I discovered that IA generated a neo-microvascular network at an incredibly rapid pace following skeletal muscle infarction. As well, I found that IA generated neocapillaries in a parallel arrangement - a hallmark of healthy skeletal muscle microvascular architecture. In addition to these favorable characteristics, IA has several critical advantages over sprouting angiogenesis. These include that 1) IA does not require the breakdown of basement membrane, 2) new vessels formed by IA are inherently perfused and thus are not required to lumenize, and 3) IA is an energy-efficient and non-proliferative process (Burri et al., 2002, Styp-Rekowska et al., 2011, Mentzer et al., 2014). Collectively, these findings suggest that optimizing IA should be exploited as a more effective and non-disruptive alternative to sprouting for rapidly re-establishing a functioning microvasculature in infarcted muscle.

5.7 Intussusceptive Angiogenesis In Ischemic Skeletal Muscle Is Based On Differential VEGFR2 Activation

The molecular divers and pathways regulating IA and pillar cell selection are poorly understood. My studies identified that VEGFR2 signaling is a regulator of the IA process during regenerative angiogenesis following skeletal muscle infarction. I identified that pillar cell selection is based on low VEGFR2 activation relative to the surrounding ECs comprising the MV wall. These findings were supported by my observation that blockade of VEGFR2 signaling in vivo increased the formation of pillars and IA in the early-stage microvasculature. In contrast, during sprouting angiogenesis, tip cell selection requires VEGF-VEGFR2 signaling wherein the EC with the highest level of VEGFR2 activation is chosen to lead the sprout (Gerhardt et al., 2003, Hellstrom et al., 2007). These findings suggest that EC intraluminal protrusion is effectively “inverse” sprouting, both structurally and with respect to the VEGF signaling axis (Figure 5.2).

I also uncovered a permissive role for ultra-low blood flow and shear forces in the formation of intraluminal pillars. This is consistent with the prediction that pillars form in local minimums of shear stress within the CAM vasculature (Lee et al., 2010). As well, my in vitro and in vivo findings indicate that ECs with low VEGFR2 activation are
Figure 5.2 Proposed model of EC inward protrusion and differential VEGFR2 activation

Top: Schematic of the cellular architecture of the mother vessel wall. Pillar-forming ECs exhibit reduced and highly focalized activation of VEGFR2 (pVEGFR2) relative to the ECs that comprise the mother vessel wall. I propose that focalized activation of VEGFR2 is present on the flow-facing side of the intraluminal protrusion or pillar. Pillar-forming ECs paradoxically sprout inwards.

Bottom: Summary and image depicting the two phenotypes of ECs found within MVs. Show in the image are two pillar-forming ECs (Type 1) on the left, and two mother vessel wall ECs (Type 2) on the right.
Pillar-Forming Endothelial Cell (Type 1)
- Rounded nucleus
- Non-aligned to flow
- Mechano-insensitive
- Flow-seeking behaviour
- Low VEGFR2 activation

Mother Vessel Wall Endothelial Cell (Type 2)
- Elongated nucleus
- Aligned to flow
- Mechano-sensitive
- Normal response to shear
- High VEGFR2 activation
unaligned to the direction of flow and I propose that they exhibit “shear-seeking” behaviour by protruding into the lumen (Figure 5.2). The goal of this would likely be to increase ligand-independent activation of VEGFR2 by blood flow shear forces. These findings arguably provide a new paradigm for the delicate interplay between the molecular and mechanical regulators of neovascularization.

5.8 Synthesis of Regenerative Angiogenesis (Chapter 3) and IA (Chapter 4) Studies

Although I discovered that IA is an effective means to rapidly generate a functioning microvasculature in ischemic skeletal muscle, I also identified that the structure of the end-stage regenerated microcirculation is highly flawed. This raises the consideration as to whether these phenomena are functionally related. IA is a high precision-process and very well proceeds imperfectly during regenerative angiogenesis in ischemic skeletal muscle. For example, primordial vessels with relatively higher shear and VEGFR2 activation in the early phases may not adequately split into a capillary mesh. If this is the case they could instead develop into AV shunts (Figure 5.3). On the other hand, other primordial vessels might split excessively and form too many capillaries. This would be consistent with the increased capillary density and branching density in the end-stage regenerated microvasculature and could be a basis for mismatch between RBC delivery and demand (Figure 5.3). Therefore, further work is warranted to understand what is required for successful regenerative angiogenesis via IA.

5.9 Future Directions in the Investigation of Intussusceptive Angiogenesis

My studies revealed a role for low VEGFR2 activation in the selection of pillar forming ECs. However, compared to the plethora of sprouting tip cell markers, the pillar-forming EC cell is not well characterized and there are no reliable immunohistochemical markers for pillars (Konerding et al., 2010). This warrants further study into the expression profile and surface markers of pillar cells that distinguish them from ECs that line the primordial vessel wall.
Figure 5.3 Steps of microvascular regeneration following skeletal muscle injury

Schematic of a healthy skeletal muscle microcirculation (top left), the loss of microvessels following femoral artery excision and muscle infarction (top right), the emergence of primordial mother vessels containing intraluminal pillars and splits (bottom left), and the end-stage and flawed regenerated microcirculation (bottom right).
Normal Microcirculation

Infarcted Muscle

Early-Stage Neovascularization

Regenerated Microcirculation

Arteriole
Venule
Venule
Capillary
Mesh
AV
Connection
1 2
3
Normal Microcirculation
Infarcted Muscle
Early-Stage Neovascularization
Regenerated Microcirculation

Femoral Artery Excision
Mother Vessel Formation
Imperfect IA
The origin of pillar-forming cells could also be explored. Transplantation of bone marrow-derived GFP-expressing cells following ischemic injury could reveal whether pillar-forming cells are circulating cells or locally-derived cells within the ischemic muscle. In addition, a “rainbow” fluorescent reporter mouse strategy could be used to assess for EC heterogeneity within primordial vessels, and for investigating whether pillar-forming ECs and MV wall ECs are distinct.

It is not understood how intraluminal pillars grow and extend down the long axis of a vessel. In this regard, an in vivo time-lapse microscopy strategy could be used to test if GFP-expressing circulating cells hone to the pillar and play a structural role in pillar expansion. Moreover, using an in vivo time-lapse microscopy strategy could reveal novel architectural cascades associated with the splitting process and potential fusion of neighbouring pillars.

EC mechanosensory pathways other than the CD31/VE-Cadherin/VEGFR2 mechanosensory complex may play a role in pillar initiation during low shear conditions. Possibilities include VEGFR3, primary cilia, caveolae, ion channels, adhesion molecules, glycocalyx, and the cytoskeleton (Coon et al., 2015, Ando et al., 2015). Establishment of an in vitro model of EC monolayers exposed to low shear forces could potentially reveal new insights into how ECs sense that they are in a low-shear environment, and how this leads to the generation of non-contact inhibitable protrusions.

Finally, my studies raise the consideration as to whether the IA process occurs in ischemic human tissues. Histological studies of patient samples, for example after amputation for peripheral vascular disease, could be performed to screen for intraluminal protrusions and pillars in microvessels.

### 5.10 Summary

Angiogenesis is critical for restoring perfusion to tissues that are attempting to regenerate following ischemic injury. For a neo-microvascular network to function properly it must be exquisitely organized, hierarchical, and have the ability to dynamically regulate O₂ delivery in order to match local metabolic requirements. As
described in this thesis, the networks that form during angiogenesis in ischemic adult tissues do not fully acquire these critical attributes. This is the case in renal tumors and in skeletal muscle following ischemic injury. Furthermore, the processes by which a neo-microvascular network regenerates in ischemic skeletal muscle occurs by the emergence of primordial vascular structures and subsequent non-sprouting intussusceptive angiogenesis and differential VEGFR2 activation. Collectively, the findings in this thesis provide novel insights into an understanding of adult angiogenesis at both a network and functional level. These novel findings prompt the reconsideration of angiogenesis paradigms and therapeutic strategies for treating ischemic disease, with the goal of generating highly functional microcirculations that reverse hypoxia.

5.11 References


Appendix A: Permission for Reproduction of Scientific Articles

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Appendix B: Ethics Approval for Animal Use

AUP Number: 2010-244  
PI Name: Pickering, Geoffrey  
AUP Title: Smooth Muscle Cells and Vascular Disease

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-244 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

**REQUIREMENTS/COMMENTS**
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Thompson, Sharla H  
on behalf of the Animal Use Subcommittee
Appendix C: Legends For Chapter 3 Video Files

**Online Video 3.1:** RBC transit in the native EDL muscle microvasculature visualized by blue light epi-illumination real-time video microscopy. RBCs are seen in relief against the bright plasma that has been labeled with high molecular-weight FITC-labeled dextran.

**Online Video 3.2:** Real-time video microscopy sequence showing no RBC transit in the EDL muscle one day following femoral artery excision.

**Online Video 3.3:** Real-time video microscopy sequence showing RBC transit signal in the early (10 day) regenerated EDL muscle microvasculature.

**Online Video 3.4:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 28 days after femoral artery excision.

**Online Video 3.5:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 56 days after femoral artery excision.

**Online Video 3.6:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 120 days after femoral artery excision.

**Online Video 3.7:** Real-time video microscopy sequence showing a neo-arteriole (top of field) in the late-stage regenerated EDL muscle microvasculature that bifurcates into daughter vessels with strikingly unequal lumen diameters.

**Online Video 3.8:** Real-time microscopy sequence of the microvasculature 28 days after femoral artery excision showing halted RBC transit.

**Online Video 3.9:** Real-time video microscopy sequence showing RBC transit (demarcated by FITC-labeled dextran) through an arteriole-capillary-venule microcirculatory unit in the native EDL muscle. A normal microvascular hierarchy is evident with divergence of an arteriole into discrete capillary meshes that drain into venules.
**Online Video 3.10:** Real-time video microscopy sequence showing RBC transit through an aberrant microcirculatory unit in the regenerated (28 day) EDL muscle microvasculature. An arteriolar-venular malformation (AV-connection) with high RBC flux can be seen to directly connect the neo-arteriole to a venule, bypassing a capillary mesh.

**Online Video 3.11:** Real-time video microscopy sequence showing RBC transit through an AV-connection in the 28-day regenerated EDL muscle microvasculature, visualized by ultraviolet light epi-illumination. A direct connection between the neo-arteriole and a venule is present.

**Online Video 3.12:** Real-time video microscopy sequence of a native EDL microvasculature showing an increase in RBC velocity and supply rate in response to a local hyperoxia challenge. The video sequence displays the identical field of interest during hyperoxia (12% O₂) and 120 seconds after converting to hypoxia (2% O₂).
Curriculum Vitae

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Education

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List of Publications


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Awards and Scholarships

• Western University Scholarship of Distinction 2006
  ○ $1,500 value

• Western University, Schulich School of Medicine and Dentistry Dean’s Honour List 2007, 2008, 2009, 2010

• Western University Graduate Research Scholarship 2010-2015
  ○ $8000 per year

• Queen Elizabeth II Graduate Scholarship in Science and Technology (QEIIGSST) 2012
  ○ $15,000 value.
• ATVB 2014 Travel Award for Young Investigators
  o $1000 value

• “Muscle Health Awareness Day 4” Ph.D. Poster Competition Prize 2014, Muscle Health Research Centre at York University
  o $150 value

• Robarts Research Institute Oral Presentation Award 2015
  o Awarded to the most outstanding PhD candidate Molecular Medicine seminar presentation out of 50 candidates
  o $100 value

• CSATVB Trainee Travel Award for ATVB 2015
  o $500 value

• NAVBO International Travel Award for IVBM 2016
  o $800 value

• Alfred Jay Medical Biophysics Award for Cellular Cardiovascular Research
  o Awarded for high quality research and associated publication
  o $1500 value

List of Presentations

• 2011 Vascular Biology Retreat
  o Poster Presentation: “Network Analysis of Angiogenesis Following Ischemia”

• 2011 Canadian Hypertension Congress
  o Poster Presentation: “Network Remodeling and Restoration of Red Blood Cell Transit in the Microvasculature During Ischemia-Induced Angiogenesis”

• 2012 Canadian Cardiovascular Congress
  o Poster Presentation: “The Robust Angiogenic Response to Hindlimb Ischemia in Mice Yields a Microvasculature That Is Structurally and Functionally Abnormal”

• 2013 Muscle Health Awareness Day, Muscle Health Research Centre at York University
  o Poster Presentation: “The Robust Angiogenic Response to Hindlimb Ischemia in Mice Yields a Microvasculature That is Structurally and Functionally Abnormal”

• 2013 North American Vascular Biology Organization (NAVBO) Conference
- Oral Presentation: “Angiogenesis Following Hindlimb Ischemia in Mice Yields a Microvasculature that is Functionally Abnormal”

- 2013 Taylor International Prize Award Symposium
  - Poster Presentation “Assessing the Function of the Regenerated Microvasculature”

- 2014 ATVB International Conference
  - Oral Presentation “Angiogenesis Following Hindlimb Ischemia in Mice Generates a Functionally Abnormal Microvasculature”

- 2014 Robarts Research Institute Retreat
  - Poster Presentation “Angiogenesis Following Hindlimb Ischemia in Mice Generates a Functionally Abnormal Microvasculature”

- 2015 ATVB and CSATVB International Conference
  - Poster Presentation “Conversion of Tumor Microvessels into a Hierarchical and Vasoreactive Network, and Suppression of Metastases, by Fibroblast Growth Factor 9”

- 2015 Robarts Research Institute Retreat
  - Oral Presentation “Regenerating the Microvasculature in Skeletal Muscle Following Ischemic Injury”

- 2016 IVBM International Conference
  - Oral Presentation “Regenerative Angiogenesis in Ischemic Skeletal Muscle Produces a Flawed Microcirculation that Poorly Controls Blood Flow”

- 2016 IVBM International Conference
  - Poster Presentation “Vascular Regeneration Following Skeletal Muscle Ischemia Occurs via Intussusceptive Angiogenesis and Differential VEGFR2 Activation”