Delineating the Skeletal Muscle-Microvessel Regeneration Program after Ischemic Injury

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Abstract

Understanding the cellular processes involved in skeletal muscle regeneration after damage is critical to advancing translational efforts toward the development of targeted therapeutics. The purpose of this thesis was to ascertain the dynamic interplay between regenerating muscle and regenerating blood vessels after ischemic injury.

First, using a novel systematic investigation of a widely used preclinical mouse model of limb ischemia, I generated a detailed atlas of muscle injury zones and angiogenesis zones in C57BL/6 mice subjected to femoral artery excision. This uncovered previously unrecognized regional variability and an exclusive relationship between angiogenesis and actively regenerating muscle zones. Then, an unbiased, systematic review of 509 manuscripts, criteria-selected from 5147 non-duplicate publications revealed that only 15% of all published studies in the field evaluated data using methods concordant with my findings. These data provide a quality assurance approach that could strengthen the value of this preclinical animal model.

Next, I tested if microvascular mural cells were a source of myogenic progenitor cells upon skeletal muscle ischemia. Using fluorescent lineage tracing techniques, I discovered that a small portion of cells marked by Myosin heavy chain-11 (Myh11) expression possessed the ability to generate new myofibers. The Myh11 lineage cell-derived myofibers were structurally indistinguishable from satellite cell-derived myofibers. Endogenous regulation of this reprogramming process was impaired by Sirtuin 6 (Sirt6) knockout, which provides considerations for future regenerative therapies.

Finally, I further examined if Sirt6 in mural cells influenced the overall regenerative response to hindlimb ischemia. Loss of Sirt6 in mural cells resulted in a necro-fibrotic phenotype that was associated with interstitial and perivascular scarring upon ischemic injury as well as medial fibrosis in larger arteries. I also discovered that the blunted muscle recovery was associated with abnormal regeneration of the microvascular network. Arteriole wrapping by smooth muscle cells was significantly reduced. The capillary density was also reduced along with loss of junctional pericytes and capillary bridges. As well, there was upregulation of
p16\textsuperscript{INK4a}, a marker of cell senescence. Together, these findings reveal a role for Sirt6 in mural cell homeostasis during muscle regeneration.

In summary, this thesis provides new insights into the relationships between the microvasculature and functional skeletal muscle regeneration after a severe ischemic insult.

\textbf{Keywords}: angiogenesis, microcirculation, ischemia, skeletal muscle, tissue regeneration, intravital microscopy, vascular smooth muscle cell, pericyte, mural cell, sirtuin 6, senescence, cellular reprogramming
Peripheral artery disease (PAD) is a vascular condition that can lead to complications like gangrene and limb amputation. Therefore, understanding this disease process is critical to the development of new treatments. The purpose of this thesis was to examine the relationship between regenerating muscles and regenerating blood vessels after vascular injury.

First, I generated a detailed atlas of muscle injury zones and zones of newly formed blood vessels in mice subjected to blood vessel injuries. This revealed a previously unrecognized geographical variability and an exclusive relationship between newly formed blood vessels and actively regenerating muscle zones. Then, I reviewed all published studies using this mouse model of disease. I discovered that only 15% of all studies in the field evaluated data using methods consistent with my findings. Therefore, my data provide a new quality assurance approach that could strengthen the value of future studies.

Next, I studied a special population of cells that attach to blood vessels, called mural cells. Here, I discovered that these blood vessel mural cells possess the ability to transform into new muscle cells, similar to a stem cell. This provides exciting new data in support of treatments that take advantage of the cells that are already in our bodies. Interestingly, a protein called Sirtuin 6 (SIRT6) that is naturally found in our bodies and decreases with age, was found to be critical to this stem-cell like potential.

Finally, I further studied the role of Sirt6 in blood vessel mural cells during muscle injury. Interestingly, the loss of Sirt6 in mural cells caused a severe injury to the muscles of affected mice. The mice without Sirt6 also suffered from more scarring after injury and the blood vessels that regenerated were abnormal. Ultimately, the blood vessel mural cells were discovered to have aged significantly beyond what was expected. This means we should seek out strategies to promote the activity of these molecules.

In summary, this thesis provides new insights into PAD and uncovers critical answers to advance regenerative medicine for this debilitating problem.
Co-Authorship Statement (where applicable)

All chapters of this thesis were written by Dr. Jason J Lee and further revised with recommendations from Dr. J. Geoffrey Pickering. Portions of this thesis have been published. The initial drafts of all manuscripts were written by Dr. Jason J Lee and the contributions of the authors of published and non-published chapters are as follows:

CHAPTER 2
Jason J. Lee, John-Michael Arpino, Hao Yin, Zengxuan Nong, Alexis Szpakowski, Abdulaziz A Hashi, Jacqueline Chevalier, Caroline O’Neil, J. Geoffrey Pickering. “Systematic Interrogation of Angiogenesis in the Ischemic Mouse Hind Limb: Vulnerabilities and Quality Assurance.” *Arterioscler Thromb Vasc Biol*. 2020 Oct; 40(10):2454-2467. Dr. Hao Yin and Dr. John-Michael Arpino contributed to the experimental design and assisted with the systematic review of the published literature. Dr. Zengxuan Nong assisted with femoral artery ligation and excision surgeries along with tissue section immunostaining. All other experimental work and analyses were performed by Dr. Jason J Lee.

CHAPTER 3
Dr. Hao Yin contributed to the experimental design. Dr. Zengxuan Nong assisted with femoral artery ligation and excision surgeries along with immunostaining. Stephanie Milkovich assisted with intravital fluorescence microscopy in live animals. All other experimental work and analyses were performed by Dr. Jason J Lee.

CHAPTER 4
Dr. Hao Yin contributed to the experimental design and with interpreting data. Dr. Zengxuan Nong assisted with femoral artery ligation and excision surgeries along with immunostaining. Ryan Wong assisted with animal breeding and genotyping. Caroline O’Neil assisted with trichrome and picrosirius red staining. All other experimental work and analyses were performed by Dr. Jason J Lee.
For Mom and Dad,
who fill my life with love, strength, wisdom, and meaning
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<tr>
<td>DAPI</td>
<td>4′,6-Di-Amidino-2-Phenyl-Indole</td>
</tr>
<tr>
<td>Ang1</td>
<td>Angiopoietin 1</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CLI</td>
<td>Critical Limb Ischemia</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo-Nucleic Acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cells</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like Factor 4</td>
</tr>
<tr>
<td>mG</td>
<td>Membrane bound GFP</td>
</tr>
<tr>
<td>mT</td>
<td>Membrane bound RFP</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotine Adenine Dinucleotide</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Artery Disease</td>
</tr>
<tr>
<td>PVD</td>
<td>Peripheral Vascular Diseases</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet Derived Growth Factor BB</td>
</tr>
<tr>
<td>Sir2</td>
<td>Silent Information Regulator 2</td>
</tr>
<tr>
<td>Sirt1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>Sirt6</td>
<td>Sirtuin 6</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor Beta 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cell</td>
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CHAPTER 1

“No man is an island, entire of itself” – John Donne

1 General Introduction

The human body, and our understanding of healthy and unhealthy states, is critically dependent on its structure and consequent function. Every cell in the human body is precisely organized into specific arrangements that form what we know as vital organs. During organogenesis, the heart and blood vessels are created first (Risau et al., 1995) and all other vital organs organize around these early blood vessels (Chan et al., 2017; Rossant et al., 2017; Shahbazi, 2020).

Postnatally, the intricate arrangements among cells can deviate from the norm, and severe deviation can cause disease. In the case of occlusive cardiovascular diseases, blood flow is severely impeded and downstream tissues are deprived of oxygen and nutrients, while local toxic waste accumulates. This results in tissue damage with consequent breakdown in structure, and ultimately the function of that organ is impaired. Peripheral artery disease (PAD) is a specific example of impaired blood flow leading to ischemic damage of downstream skeletal muscles. In its most severe clinical manifestation, critical limb ischemia (CLI) carries high risk of chronic wound, limb amputation and even death (Farber, 2018; Farber et al., 2016).

Upon injury or disease, there is an attempt to repair and/or regenerate the damaged tissue back to a state that closely mimics the original structure to restore optimal function. To better understand this recovery process, the interplay between the regenerating vascular network and the regenerating end-organ, including skeletal muscle, needs to be explored. Currently, how the vascular system coordinates skeletal muscle regeneration is poorly understood. The concept of therapeutic angiogenesis as the nidus for robust repair of injured muscle has shown promise in experimental models but, failed to produce clinical benefit for patients with PAD (Annex, 2013; Iyer et al., 2017; Simons et al., 2003).
To advance our understanding of the optimal muscle repair and regeneration process, it is important to understand the mechanisms of this translational failure. Furthermore, novel insights into the cellular components of the vascular network along with their phenotypic and functional plasticity – in the context of ischemic damage – is important to revealing new avenues of PAD treatment. Finally, to therapeutically leverage any innate regenerative potential within the vascular network, new molecular targets that modulate the relationship between the regenerating microvasculature and the regenerating skeletal muscle need to be uncovered.

The broad goal of my research was to ascertain the interplay between the regenerating skeletal muscle and the regenerating vascular network, following ischemic injury. I further sought to identify novel roles and regulatory cascades of vascular mural cells in skeletal muscle damaged by ischemia. To address these broad research goals, I have pursued three specific objectives:

1. To define the skeletal muscle injury and angiogenesis landscapes in mice subjected to hindlimb ischemia, and determine if published studies to date have used an analysis strategy concordant with these landscapes.

2. To determine if vascular mural cells give rise to regenerated skeletal myofibers in adult mice subjected to hindlimb ischemia.

3. To determine if deleting the sirtuin enzyme, Sirtuin 6 (Sirt6), in vascular mural cells compromises the regenerative process in mice subjected to hindlimb ischemia.

1.1 Development of the Healthy Skeletal Muscle

1.1.1 Embryogenesis of Skeletal Muscle

The formation and development of all embryos, including the human embryo, is a highly conserved coordinated process (Chan et al., 2017; Rossant et al., 2017; Shahbazi, 2020). What begins with fertilization is quickly followed by cleavage, morula compaction and differentiation that produces the blastocyst (Standring, 2016). The free floating blastocyst,
with the blastocoele and inner cell mass wrapped within the trophectoderm, eventually implants into the mother’s endometrial lining within the uterus (Rossant et al., 2017). Once implanted, the embryonic disc undergoes gastrulation, which forms the three germ layers known as ectoderm, mesoderm, and endoderm. The formation of the three germ layers signal the beginning of neurulation and organogenesis (Standring, 2016).

Skeletal muscle is derived from the presomitic paraxial mesoderm, which eventually forms somites (Buckingham et al., 2003; Chal et al., 2017). Within the somites, premyogenic precursors and skeletal myoblasts are characterized by their *Pax3* expression (Buckingham et al., 2003; Chal et al., 2017). During development, these *Pax3*+ progenitor cells migrate to the limb bud where they proliferate, differentiate and fuse to form mature myofibers. At this stage, a subset of *Pax3*+ myogenic progenitors also begin to downregulate *Pax3* expression and begin to express *Pax7* – a marker of adult satellite cells (Chal et al., 2017). In the adult, these *Pax7*+ myogenic progenitor cells are responsible for myogenesis during muscle regeneration after injury (Lepper et al., 2011; Sambasivan et al., 2011).

### 1.1.2 Embryogenesis of the Vasculature

Vasculogenesis and angiogenesis are the formative processes by which new blood vessels and vascular networks are produced. In the human embryo, vasculogenesis occurs via the differentiation of vascular precursor cells, known as angioblasts, into mature endothelial cells that self-organize to generate vascular structures (Carmeliet, 2000; Risau, 1997; Risau et al., 1995). Angioblasts originate from the mesodermal layer, which depend on endoderm-derived factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), to form luminal structures resembling blood vessels (Risau et al., 1995).

Once a primitive vasculature is formed, it undergoes maturation and remodeling to produce a functional hierarchical vascular network. This process, termed vascular myogenesis, requires the investment of mural cells, which encompasses vascular smooth muscle cells (VSMCs) and pericytes (Carmeliet, 2000). This growth and maturation is critical to optimal function as the loss of mural cells have been shown to cause vessel regression or pathological vessel structures (Carmeliet, 2000). Therefore, various factors like platelet
derived growth factor BB (PDGF-BB), Angiopoietin 1 (Ang1) and transforming growth
factor beta 1 (TGF-β1) help to recruit mural cells to the premature vascular plexus and
strengthen their interaction (Carmeliet, 2000). Interestingly, unlike endothelial cells, which
appear to originate from their own embryologic source that invade and interact with other
primitive organ tissues for maturation, VSCMs are in fact locally derived (Bargehr et al.,
2016; Cheung et al., 2012; Majesky, 2007; Schwartz et al., 1990). Therefore, the pool of
VSMCs and pericytes throughout the human body have unique embryological origins with
functional consequences (Bargehr et al., 2016; Sawada et al., 2017). In skeletal muscle,
pericytes – which also originate from the paraxial mesoderm and somites – contribute to
not only vascular network development and maturation, also is thought to participate in
muscle regeneration and fibrosis (Cappellari et al., 2013).

1.1.3 The Role of the Vasculature During Organ and Skeletal
Muscle Development

Studies have shown that there exists a two-way communication between endothelial cells
and organ-specific cells in developing tissues, which directs organ development. This
critical crosstalk has been studied in various vital organs such the lungs, liver, and pancreas
(Cleaver et al., 2012; Cleaver et al., 2003; Lazarus et al., 2011; Nikolova et al., 2003). In
these organs, the disruption of endothelial cell signaling to target tissues has been shown
to produce deleterious results during development (Cleaver et al., 2003; Nikolova et al.,
2003).

Another emerging function of the vasculature in the development, maintenance and
regeneration of organ tissues is its role in tissue-specific resident stem cell and progenitor
cell maintenance (Cleaver et al., 2012; Cleaver et al., 2003; Nikolova et al., 2007). Studies
have uncovered a microenvironment, termed the ‘vascular niche’, where stem cell and
progenitor cell populations are preserved for potential future activation. The vascular niche
has been proposed to include not only the endothelial and mural cells of the vascular tree,
but also the associated basement membrane and extracellular matrix (Nikolova et al.,
2007). In fact, much of the interaction between the vascular niche and the multipotent cells
is thought to be through recruitment of the vasculature by target organ cells, usually via
VEGF, followed by vascular basement membrane-associated signaling (Cleaver et al.,
2012; Nikolova et al., 2007). These data further emphasize the importance of understanding the tight structural and physiological interactions between the cells of the vasculature and associated end organs, not only in development, but also in adulthood diseases like PAD (Figure 1.1).

1.2 The Unhealthy State of Peripheral Vascular Disease

If the healthy state of human biology is considered to be the appropriate organization and structure of cells that enables optimal tissue function, then the unhealthy state is simply the disruption of this organization and structure that results in poor function.

Peripheral artery disease (PAD), also referred to as peripheral vascular disease (PVD), is an ischemic disease caused by systemic atherosclerosis that reduces blood supply to the extremities. In its most severe form, CLI can lead to chronic skin ulcers, gangrene, and limb amputation. Its prevalence increases with age and has the second fastest growing mortality rate globally, behind only atrial fibrillation/flutter (Lozano et al., 2012; Song et al., 2019). Between 1990 and 2010, PAD mortality rates increased by 167%; during the same time period, ischemic heart disease grew by 35% and cerebrovascular disease by 26% (Lozano et al., 2012; Song et al., 2019). Interestingly, PAD is distinct from cardiac and cerebral vascular diseases due to the ability of skeletal muscles to regenerate. This regenerative capacity raises prospects for therapeutically reversing tissue damage and restoring tissue structure and function.

This injury and regeneration response is the result of many complex underlying mechanisms such as genetic and epigenetic issues, protein translation and post-translational modifications, and external environmental interference. To fully understand the fundamental pathways of disease and disease response, effective use of animal models may facilitate efficient knowledge discovery. In this regard, there are several different models of skeletal muscle injury and regeneration that enable detailed investigations of the muscle injury response.
Figure 1.1 The Interdependent Cellular Relationships within Healthy and Regenerating Tissues

The tissue microvascular unit is comprised of end-organ structures like skeletal myofibers tightly coupled to an accompanying vascular network that consists of arterioles, capillaries, and venules. The crosstalk between the various cells within this microenvironment dictate the maintenance health states as well as response to injury.
1.3 Models of Skeletal Muscle Injury and Regeneration

Studies using mouse models of skeletal muscle regeneration after injury have yielded vital information regarding the relevant genetic and molecular pathways (Maden et al., 2020). The speed of recovery and muscle regeneration may vary according to the type of injury (Hardy et al., 2016) and the genetic makeup of the mouse, but function usually returns by 28 days following tissue damage (Arpino et al., 2017; Maden et al., 2020). In certain cases, muscle regeneration may still occur after repeated insults (Maden et al., 2020).

Animals models of skeletal muscle injury and regeneration achieve tissue damage by either vascular disruption causing muscle ischemia (Couffinhal et al., 1998) or exposure to myotoxic stimuli (Hardy et al., 2016). In models that employ direct myotoxic stimuli, such as barium chloride-induced muscle injury (Guimaraes-Camboa et al., 2017) or cardiotoxin-induced injury (Dellavalle et al., 2011), the muscle injury responses are susceptible to toxin-induced side effects on myogenic progenitor cells and the vasculature (Hardy et al., 2016). Indeed a comparison of the various models of skeletal muscle injury and regeneration showed that both chemical injury by barium chloride as well as cardiotoxin induced-injury caused significant loss of satellite cells (Hardy et al., 2016). Therefore, induction of injury that avoids toxin-induced cellular injury side effects may offer a more accurate model of the ischemia-induced muscle injury seen in patients with PAD.

1.3.1 Hindlimb Mouse Model of Ischemic Skeletal Muscle Injury

Of the established models of tissue regeneration, the rodent mouse model most closely resembles human tissues, both in structure and physiology (Ericsson et al., 2013; Maden et al., 2020; Phipps et al., 2020). Within the realm of mouse models of tissue regeneration, one of the most widely used, especially in the field of vascular medicine, is the hindlimb mouse model of ischemic skeletal muscle injury, first established by Couffinhal et al. in 1998 (Couffinhal et al., 1998). In this model, ischemia is induced by ligation and excision of the femoral artery, which severely restricts blood supply to the affected hindlimb. This mouse model is particularly interesting for not only its skeletal muscle regeneration, but also for the regeneration of the injured vascular network. Since the original publication, thousands of studies have used this model of vascular disease and muscle regeneration to
better understand human vascular pathologies like PAD (Annex, 2013; Iyer et al., 2017). The innate regenerative mechanism, coupled with genetic tools for careful isolation of key biological effectors, afford an excellent opportunity for highly informative research.

1.3.2 Skeletal Muscle Regeneration

The myocytes and myofibers that make up the mature skeletal muscle are embryologically derived from the mesoderm (Charge et al., 2004; Ten Broek et al., 2010). In the adult skeletal muscle, damaged myofibers have the natural ability to regenerate through resident progenitor cells known as satellite cells (Charge et al., 2004). Satellite cells are identified by their cellular specific marker Pax7 (Chal et al., 2017). These cells are often found scattered on across the surface of mature skeletal myofibers and in close proximity to the microvascular network (Ten Broek et al., 2010). Interestingly, recent studies have identified other cell populations with multipotent progenitor properties, including vascular pericytes (Cappellari et al., 2013; Dellavalle et al., 2011; Ten Broek et al., 2010), although their innate ability to regenerate tissue in vivo still needs to be clarified (Guimaraes-Camboa et al., 2017).

1.3.3 Collateral Recruitment and Angiogenesis

The mouse model of skeletal muscle injury also provides opportunities to evaluate the regeneration of blood vessels after insult, as well as the interaction between the vascular network and the downstream tissues it supplies (Simons et al., 2003). This process begins with the diversion of blood flow into pre-existing collateral vessels that bypass points of arterial occlusion (Heil et al., 2006). The maturation of collateral arteries, also referred to as collaterogenesis and arteriogenesis, requires the proliferation of vascular endothelial cells and mural cells for luminal expansion and vessel wall muscularization (Deindl et al., 2001; Gifre-Renom et al., 2021; Kondoh et al., 2004). This remodeling process facilitates the delivery of bulk blood flow to ischemic muscles beyond the point of native artery occlusion (Limbourg et al., 2009). Thereafter, in the hindlimb ischemia mouse model of PAD, angiogenesis restores the microvascular network near the uninjured state by 14 days following injury (Arpino et al., 2017).
Studies have found that the mere presence of a regenerated vascular network does not equate to a fully normalized, functional vascular network (Arpino et al., 2017). In fact, Arpino and colleagues discovered that the regenerated microvascular network is flawed in structure as well as function, and does not remodel or improve over time. Despite the robust regeneration of the vascular bed, the hierarchical order between arteriole, capillary and venule can be lost. Also, mural cell wrapping, which not only supports the endothelial cells, but also helps control blood flow in response to demand, is patchy and inconsistent. Therefore, further work is needed (Iyer et al., 2017) to understand the barriers in regenerating a structurally normal, fully functional vascular network that can robustly support downstream tissues. Moreover, in order to clinically translate vascular regenerative potentials into benefit for patients with disease, the coordination between vascular regeneration and surrounding tissue regeneration will need to be elucidated.

1.3.4 Cellular Lineage Tracing

To understand how the cells of the microvasculature navigate through a diseased or injured tissue toward repair and regeneration, it is important to track the progress of a cell or group of cells through its transformations. Lineage tracing and fate mapping can be performed in transgenic mouse models as well as in some human tissues via various established systems (Bentzon et al., 2018). Lineage tracing and mapping relies on the permanent labelling of a cell or cells of interest, which can be later combined with other cell identification methods such as immuno-histologic protocols. Here, permanent labelling of specific cells can be performed via several different strategies (Bentzon et al., 2018). Historically, early lineage tracing studies relied on the expression of a β-galactosidase reporter transgene. Later, xenotransplantation and sex mismatched tissue transplantation methods capitalized on naturally occurring differences in the target cells of interest compared to the host (J. Li et al., 2001). More recently, various combinations of fluorescent protein expression strategies along with naturally occurring epigenetic cellular signatures have proven to be powerful tools in research, both in animals models as well as in human patients (Bentzon et al., 2018; Cattaneo et al., 2020; Gomez et al., 2012; Shankman et al., 2015). For example, using fluorescent lineage tracing strategies in transgenic mice, Liu and colleagues, demonstrated the existence of Twist2+ myogenic progenitor cells that are distinct from Pax7+ satellite...
cells (N. Liu et al., 2017) in the regenerating adult skeletal muscle. Therefore, future studies that aim to examine the interaction between plastic cellular components of the vasculature and regenerating tissues is likely to require the employment of these powerful systems.

1.4 The Vascular Mural Cells in Atherosclerotic Vascular Diseases and Skeletal Muscle Regeneration

In addition to their structural supportive role within the vascular network, vascular mural cells also possess plasticity. Since the 1990s, human VSMCs have been shown to reversibly convert between contractile and noncontractile phenotypes (S. Li et al., 1999). More recent studies have confirmed that during development and disease, VSMCs can transdifferentiate into other cell types such as stem-like cells, macrophage-like cells and myofibroblasts (Alencar et al., 2020; Feil et al., 2014; Gomez et al., 2012; Owens et al., 2004; Shankman et al., 2015; Sorokin et al., 2020). Pericytes, which are concentrated at the microvascular capillary beds, have similarly displayed tantalizing multipotent potential (Nwadozi et al., 2020). Therefore, when investigating the regeneration of muscle tissues towards the normal structural and functional state, the roles of the mural cells must be considered.

1.4.1 Vascular Smooth Muscle Cells in Atherosclerosis

Atherosclerosis, characterized by its hallmark atherosclerotic plaque, is a dynamic and complex phenomenon that involves the accumulation of excess lipids at the subendothelium, followed by inflammation and vessel wall remodeling (Hansson et al., 2011; Wolf et al., 2019). Systemic atherosclerosis is the underlying pathologic basis for arterial occlusion in PAD. Within this pathogenic process, the VSMC was thought to play a minor role, especially in the inflammatory response seen within plaque lesions. However, recent evidence suggests a more active role that highlights the importance of its phenotypic plasticity. Specifically, it has been shown in human atherosclerotic plaques of the aorta that VSMCs have the ability to transdifferentiate into macrophage-like cells, myofibroblasts and even mesenchymal stem-like cells, in a Kruppel-like factor 4 (KLF4) dependent manner (Shankman et al., 2015; Sorokin et al., 2020). Up to 30% of macrophage cells found in plaque lesions were found to be of VSMC origin (Shankman et al., 2015). What is still yet to be determined is the extent to which VMSCs contribute to the atherosclerotic
lesions in PAD, their involvement in the initiation of disease pathogenesis, and the 
regulatory cascades involved in VMSC plasticity amenable to targeted therapy.

1.4.2 Pericytes in Disease and Muscle Regeneration

In addition to VSMCs, pericytes, also known as Rouget cells, make up the other component 
of the vascular mural cell population in the body. In certain organs, such as the kidney and 
liver, pericytes have tissue specific names - mesangial cells and hepatic stellate cells 
respectively – which have been shown to serve specialized functions (Shaw et al., 2018; 
Trivedi et al., 2021). In addition to their contractile mural cell role within vascular 
networks, pericytes have long garnered interest for their mesenchymal stem-like properties 
and their multipotent potentials (Crisan et al., 2008; Nwadozi et al., 2020). Most intriguing 
is the body of evidence accumulating for pericytes and their implicated roles in both disease 
pathogenesis (Cappellari et al., 2013; Ray et al., 2020) as well as tissue repair and 
regeneration (Cappellari et al., 2013; Nwadozi et al., 2020).

Pericytes as therapeutic targets for skeletal muscle regeneration have been actively pursued 
(Crisan et al., 2008; Dellavalle et al., 2011) due to their multipotent stem-like properties as 
well as their ubiquity throughout the human body. In 2008, Crisan et al. demonstrated the 
myogenic potential of human skeletal muscle pericytes in culture (Crisan et al., 2008). 
Then, using a mouse model with pericytes transgenically labelled with an inducible 
alkaline phosphatase-CreERT2, Dellavalle et al. provided in vivo evidence of the myogenic 
potential of pericytes during post-natal development (Dellavalle et al., 2011). However, 
direct in vivo evidence has been lacking in the adult skeletal muscle after injury and clinical 
utility has been debated (Guimaraes-Camboa et al., 2017). Fully elucidating the 
multipotent potential of pericyte populations will be important for disease understanding 
and future therapeutics.

1.5 Mural Cell Plasticity and Cell Metabolism

From embryogenesis to adulthood, pluripotent stem cells and multipotent stem-like cells 
have held considerable interest for their unique abilities for self-renewal as well as 
therapeutic potential for tissue regeneration. It was not until 2006 when Yamanaka et al.
published their report describing the induction of somatic cell dedifferentiation into pluripotent stem cells that pluripotency and stemness was accepted as a bidirectional state that could be manipulated (Takahashi et al., 2006). Since then, numerous additional studies have discovered that cellular energy metabolism is a critical facilitator of nuclear reprogramming (Folmes et al., 2011; Tsogtbaatar et al., 2020). Specifically, reprogramming the somatic cell’s metabolic machinery from oxidative phosphorylation to a glycolysis dependent state in the stem cell, and vice versa, is central to this process (Folmes et al., 2011; Tsogtbaatar et al., 2020). Consequently, in the context of the vascular network and the role of plasticity in mural cells during disease and tissue regeneration (M. Liu et al., 2019; Majesky et al., 2017; Nwadozi et al., 2020), the same concepts likely drive the biological phenomena.

1.5.1 Sirtuin Histone Deacetylases

To date, numerous studies have confirmed a tight association between the cell’s metabolic profile and epigenetic regulation of downstream processes (Harvey et al., 2019; Ryall et al., 2015). Broad categories of relevant epigenetic regulation include histone acetylation, histone methylation and DNA methylation (Harvey et al., 2019; Ryall et al., 2015). Deeper analysis into the regulatory proteins that control these pathways has revealed a distinctively conserved family of enzymes called sirtuins, which regulates histone acetylation via nicotinamide adenine dinucleotide (NAD) dependent protein deacetylation. The sirtuin enzyme family, homologues of the Silent Information Regulator 2 (Sir2) domain, is a seven member group (Sirtuin 1-7) that is highly conserved from bacteria to humans (Frye, 2000).

The sirtuin family of proteins is additionally intriguing in their in vivo biological effects on aging, including longevity (Kaeberlein et al., 1999). It was shown by Kaeberlein et al. that sirtuin enzymes significantly increase the lifespan of yeast cells and, conversely, critically shorten lifespan in their absence. Shortly thereafter, sirtuin enzymes were discovered to be NAD-dependent histone deacetylases by the same group (Imai et al., 2000). In the short time since this seminal discovery in 2000, all seven members of the sirtuin family have been extensively studied. Specifically in relation to cellular metabolism and multipotency, every member of the sirtuin family has been reported to be involved in stem cell differentiation and pluripotent stem cell formation (Hsu et al., 2018). Of the sirtuins, Sirtuin
1 (Sirt1) has been most studied and has been shown to both facilitate in the induction of pluripotency as well as regulate differentiation of stem cells via various mechanisms (Calvanese et al., 2010; Han et al., 2008; Hsu et al., 2018; Lee et al., 2012). Of note, Sirt1 has also been shown to regulate the expression and activity of other sirtuins including sirtuin 6 (Sirt6) (Kim et al., 2010; Meng et al., 2020) via complex formation at the Sirt6 promoter and direct deacetylation of the Sirt6 protein itself.

1.5.2 Sirtuin 6

Nuclear sirtuin protein Sirt6 is intriguing among all sirtuins because of the striking phenotype of the knockout mouse model (Mostoslavsky et al., 2006), primate models of disease (W. Zhang et al., 2018) as well as human findings of lethal deleterious effects (Ferrer et al., 2018). In the mouse model, pups born with a Sirt6 deficiency were shown to have severely stunted growth, spontaneous organ inflammation and death by hypoglycemia by 28 days of age. Since this seminal publication in 2006, many others have identified various new roles and novel mechanisms by which Sirt6 plays vital roles in health and disease (Chang et al., 2020; Tasselli et al., 2017). These include DNA repair and damage response, telomere maintenance, regulation of cell metabolism including glycolysis and gluconeogenesis, lipogenesis and lipolysis, regulation of inflammation, regulation of tumor suppression and stem cell regulation (Chang et al., 2020; Tasselli et al., 2017).

1.5.2.1 Sirtuin 6 and Aging

The effects of Sirt6 on aging have been studied in both abundant and deficient states (Chang et al., 2020; Lombard et al., 2008; Tasselli et al., 2017). When Sirt6 was overexpressed in male mice, it increased lifespan by a mean of approximately 15% (Kanfi et al., 2012). This added longevity was attributed to alterations in insulin-like growth factor 1 (IGF1) signaling (Kanfi et al., 2012). In Sirt6 knockout mice, there was an accelerated progeroid effect resulting in death by 28 days of age (Mostoslavsky et al., 2006). At the molecular level, the effects of Sirt6 on aging and senescence has been shown to be multifactorial (Chang et al., 2020; Lombard et al., 2008; Mostoslavsky et al., 2006), including regulation of telomere metabolism (Michishita et al., 2008), inflammation (Jiang et al., 2013; Kawahara et al., 2009), autophagy (Chen et al., 2018), and TGFβ induced...
senescence (Minagawa et al., 2011). Even in self-replicating multipotent cells like bone
marrow mesenchymal stem cells, knockdown of Sirt6 in result in expression of senescence
associated β-galactosidase and p16$^{Ink4a}$ (Zhai et al., 2016). The role of Sirt6 in senescence
of vascular mural cells within the ischemic skeletal muscle has not yet been investigated.

### 1.5.2.2 Sirtuin 6 and TGFβ Signaling

Interestingly, Sirt6 has also been shown to post-translationally regulate TGFβ signaling via
deacetylation of Smad2 and Smad3 transcription factors, which suppress their activity
(Mainty et al., 2020; J. Zhang et al., 2021; Zhong et al., 2020). In the absence of Sirt6, Smad3
acetylation may be increased, which could result in increased TGFβ signaling, resulting in
tissue fibrosis (Maity et al., 2020). Furthermore, Sirt6 deacetylase activity at key histone
sites have also been shown to downregulate TGFβ signaling genes (Maity et al., 2020; J.
Zhang et al., 2021; Zhong et al., 2020). During skeletal muscle regeneration, TGFβ
signaling has been shown to inhibit myoblast proliferation (Delaney et al., 2017; X. Xu et
al., 2018). In the aorta, downregulation of TGFβ signaling is crucial for VSMC
collectivization and vessel integrity (Balint et al., 2015).

### 1.5.2.3 Sirtuin 6 and Cell Plasticity

In the realm of stem cell homeostasis, Sirt6 has been shown to play a critical role in gene
regulation. In the healthy state, pluripotency factors like Oct4, Sox2, and Nanog are
repressed via H3K56 deacetylation to allow stems cells to differentiate (Carey et al., 2014;
Kashyap et al., 2009; Tan et al., 2013; Xie et al., 2009). In the absence of Sirt6, the
hyperacetylated state leads to sustained expression of pluripotent genes, which ultimately
blocks stem cell differentiation (Etchegaray et al., 2015; Jain et al., 2016).

Studies have also shown a consistent defect in the reprogramming potentials of Sirt6-
deficient differentiated adult cells (Sharma et al., 2013; P. Xu et al., 2019). In aged human
dermal fibroblast cell lines that natively express SIRT6 at low levels, SIRT6 deficiency
was associated with reduced reprogramming efficiency that was rescued by delivery of
exogenous SIRT6 (Sharma et al., 2013). However, the exact mechanisms of the effects of
Sirt6 on cellular reprogramming are still unknown.
Further detailed characterization and identification of therapeutic targets within the sirtuin pathways will be critical for future endeavors that examine the relationship between cellular metabolism and vascular mural cells during muscle regeneration after ischemic injury.

1.6 Therapeutic Regeneration of Healthy Tissues

The ultimate goal of clinical therapy and therapeutics is to return the patient who suffers from disease back to the healthy state, as much as possible. There are many approaches to this goal with varying likelihood of success. But, the most ideal approach may be to return the body’s diseased structure back to the original healthy form. To aim for such a feat requires understanding the biological components of a healthy tissue and the timely interaction among them. If the embryological process of human development is taken as a template for tissue generation and regeneration, the communication between the vascular network, the cells within the vascular niche and the end organ tissues may hold an enticing promise that should be explored.

1.6.1 The Translational Valleys of Death

In an effort to return the body’s diseased structures back into their original form, a therapeutic strategy called therapeutic angiogenesis has been extensively studied, particularly in the clinical field of PAD (Annex, 2013; Iyer et al., 2017; Simons et al., 2003). Here, the diseases are typically characterized by disruption of normal blood flow past a point of blockage within the vascular tree. In order to regenerate damaged downstream skeletal muscles, attempts have been made to regenerate a functional vascular tree with the goal of returning blood flow and begin the regeneration process (Iyer et al., 2017; Simons et al., 2003). Many pre-clinical animal studies have shown potential for benefit using this approach (Iyer et al., 2017). However, all attempts at therapeutic angiogenesis in clinical settings have failed (Iyer et al., 2017; Simons et al., 2003). This represents what is now known as the Translational Valleys of Death (Butler, 2008; Reis et al., 2008).
The Translational Valleys of Death refer to the disconnect between knowledge gained from pre-clinical studies and the clinical application of those information. In order to achieve the goal of health regeneration and rejuvenation, the factors that contribute to this translational gap will also need to be addressed in future studies.

1.7 Aims of the Thesis

The return of skeletal muscle health after ischemic insult is a process that depends on skeletal myofiber regeneration from progenitor cells, restitution of blood flow, arteriogenesis, angiogenesis, and vascular remodeling. The dynamic relationship between neovessel formation and tissue regeneration is also interdependent and tightly linked.

The overall hypothesis of my thesis is that skeletal myogenesis and angiogenesis following ischemic injury are irrevocably linked.

I further hypothesize that microvascular mural cells – smooth muscle cells and pericytes – are critical to a productive regenerative response to ischemic skeletal muscle injury.

I present my data in three Chapters:

**Chapter 2**: My objective was to determine the spatial distribution and spatial relationships of skeletal muscle injury and angiogenesis in the mouse hindlimb subjected to a severe ischemic insult. For this, I undertook femoral artery excision in the mouse hindlimb and mapped the spatial profile landscapes for the entire hindlimb. I also determined whether the current large amount of published data on angiogenesis following hindlimb ischemia used an analysis strategy concordant with my findings. This was performed via a high-content systematic review and evaluation.

**Chapter 3**: My objective was to determine whether pericytes can give rise to regenerated skeletal muscle myofibers after an ischemic insult. Using genetically labelled fluorescent mouse models, I undertook femoral artery excision in the mouse hindlimb and performed cell lineage tracking using live in vivo imaging and fluorescence microscopy analyses.

**Chapter 4**: My objective was to determine the consequences of genetic ablation of SirT6 in vascular mural cells on skeletal muscle repair and regeneration in the mouse hindlimb.
subjected to a severe ischemic insult. For this, I generated a smooth muscle myosin heavy chain \textit{Myh11}-dependent inducible mouse model of \textit{Sirt6} deletion in mice in which the deletion event can be identified based on conversion of a fluorescence signal.

Collectively, my thesis provides new data on the dynamic interplay between regenerating microvessels and skeletal muscles subjected to ischemic injury.

1.8 References


Influences Their Ability to Support Endothelial Network Formation. Stem Cells Transl Med, 5(7), 946-959. doi:10.5966/sctm.2015-0282


CHAPTER 2

2 Systematic Interrogation of Angiogenesis in the Ischemic Mouse Hindlimb: Quality Assurance and the Translational Valley of Death


2.1 Introduction

Peripheral vascular disease is a worldwide health burden (Collaborators, 2015; Fowkes et al., 2013). Its most serious manifestation, critical limb ischemia, carries high risks of limb amputation and death, despite advances in percutaneous and surgical therapies (Farber, 2018; Farber et al., 2016). Accordingly, treatment innovations for peripheral artery disease are actively investigated, including strategies to augment angiogenesis.

A common approach to investigating ischemia-induced angiogenesis is a mouse model of hind limb ischemia. In 1998, Couffinhal et al (Couffinhal et al., 1998) reported such a model wherein blood flow down the femoral artery was surgically halted, leading to neovascularization of the hind limb skeletal muscles. This experimental system has since been adopted widely to study ischemia-induced angiogenesis and evaluate pro-angiogenesis therapies (Annex, 2013; Vale et al., 2001). Drugs, hormones, growth factors, progenitor cells, and biomaterials have been tested, many with positive findings (Hisaka et al., 2004; Masaki et al., 2002; Morishita et al., 2002; Said et al., 2016; Silvestre et al., 2003; Suzuki et al., 2012). This, in turn, has informed paradigms for patient intervention (Annex, 2013; Iyer et al., 2017; Rigato et al., 2017). However, despite preclinical successes in the mouse, no pro-angiogenesis interventions have proven to benefit patients with peripheral vascular disease (Belch et al., 2011; Iyer et al., 2017; Powell et al., 2008; Rajagopalan et
This model-patient discordance constitutes an example of a translational valley of death (Butler, 2008; Reis et al., 2008).

There are several potential explanations for the discordance between the preclinical and clinical outcomes. These include the older age and coexisting chronic diseases of individuals with peripheral artery disease (Lotfi et al., 2013; Nowak-Sliwinska et al., 2018), challenges in scaling local delivery strategies for humans (Annex, 2013; Baumgartner et al., 2009), insufficient inclusion of female mice, and the fact that new microvessels may not acquire the necessary complement of vasomotor functions (Arpino et al., 2017; Frontini et al., 2011; R. K. Jain, 2003).

In addition to these considerations, it must also be recognized that the histological evaluation required to assess angiogenesis could, itself, compromise data reliability. Angiogenesis is a microscopic process, yet the skeletal muscle territories in the hind limb are vast (Charles et al., 2016; Kochi et al., 2013). This challenges accurate data extraction, particularly with regional heterogeneity in angiogenesis, as may arise from different skeletal muscle metabolic subtypes (Cherwek et al., 2000; Zaccagnini et al., 2015) and variable collateral responses (Zbinden et al., 2007). Different mouse strains and different interventions used to induce ischemia can further complicate the sampling-based evaluation strategy (Helisch et al., 2006; Limbourg et al., 2009). Appropriately selecting microscopic territories from the immense muscle landscape is critical not only for consistent results but also accurate conclusions. For example, inadvertent mismatching of muscle zones for treatment and control intervention groups could lead to concluding the existence of a treatment effect where none exists. Given these concerns, it is noteworthy that a comprehensive understanding of where angiogenesis proceeds in the ischemic hind limb, and its relationship to skeletal muscle pathobiology, is lacking.

Herein, we have undertaken a hybrid, experimental-evaluative investigation of the vulnerabilities in a widely used preclinical mouse model of limb ischemia, using systematic approaches. First, we generated a detailed atlas of muscle injury zones and angiogenesis zones across 22 hind limb muscles of C57BL/6 mice subjected to femoral artery excision. Second, we undertook an unbiased, systematic review of 509 manuscripts, criteria selected
from 5147 non-duplicate publications of mouse hind limb ischemia. From these manuscripts, we ascertained the extent to which published investigations used an analysis approach concordant with the mapping data. The findings uncover substantial data risks for mouse hind limb ischemia studies and provide a quality assurance approach that could strengthen the value of this preclinical animal model.

2.2 Materials and Methods

2.2.1 Mouse Model of Hind Limb Ischemia

Experiments were conducted in accordance with the University of Western Ontario Animal Care and Use Subcommittee, which follows the policies set out by the Canadian Council on Animal Care. This study was also conducted in accordance with the Animal Research: Reporting In Vivo Experiments guidelines for transparent reporting of animal research (Kilkenny et al., 2010) and the 2018 consensus guidelines for the use and interpretation of angiogenesis assays (Nowak-Sliwinska et al., 2018). C57BL/6J (Jackson Laboratories, Bar Harbor, ME) and C57BL/6N (Charles River Laboratories, Wilmington, MA) mice 12 weeks of age were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Male mice were studied to reflect the currently used methodologies in the literature and recognizing their robust angiogenic response (Peng et al., 2011). Hind limb ischemia was induced by ligating the right femoral artery above and below the profunda femoris branch, recently re-termed the proximal caudal femoral artery (Kochi et al., 2013), using 6-0 silk sutures and excising the intervening 2- to 3-mm portion of artery (Frontini et al., 2011; Limbourg et al., 2009). All hind limb muscles were harvested after 10 days, a time point in which capillary regeneration is known to be robust, and after 28 days, when the process has stabilized (Arpino et al., 2017). Mice were housed in clear, plastic cages containing standard bedding and maintained on a 12-hour light and dark cycle at 23°C and 50% relative humidity. Mice were fed water and normal mouse chow diet (Diet 2018; Harlan Teklad, Madison, WI), which were available ad libitum.

2.2.2 Laser Speckle Contrast Imaging Flow Analysis

Bulk blood flow to the distal hind limb was assessed using laser speckle contrast imaging (moorFLPI-2; Moore Instruments, United Kingdom). Relative limb perfusion was
measured pre- and post-ischemia using regions of interest confined to the plantar soles, as reported previously (Limbourg et al., 2009). Mice were lightly anesthetized using isofluorane (1.3%–1.5% mixed with 100% oxygen at a rate of 0.8 L/min) and placed on a heating pad, covered by a black mat provided by the manufacturer, to maintain a body temperature of 36.5±0.5°C. The mice were acclimatized to the anesthetic and heating pad for 5 to 7 minutes before imaging and flow data collected according to manufacturer instructions. Image settings were set to Temporal Measure Mode with a Temporal Filter of 250 frames. Postprocessing of images and data extraction were performed using moorFLPI Review V5.0 software (Moore Instruments). Results are expressed as the ratio of perfusion in the ischemic (right) limb versus the nonischemic (left) limb. Mice with postoperative day 0 perfusion ratios that did not decline to 30% of baseline or lower were excluded, in line with currently accepted standards (Limbourg et al., 2009) and allowing for the higher spatial resolution of laser speckle contrast imaging compared with laser Doppler imaging (Figure 2.1).

2.2.3 Tissue Preparation

For tissue harvesting, mice were subjected to isofluorane overdose and perfused sequentially with PBS and 4% paraformaldehyde via left ventricular cannulation at physiological pressure. Whole left and right hind limbs were dissected, immersed in 4% paraformaldehyde overnight, and then decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days (Arpino et al., 2017). Each hind limb was embedded in paraffin and sectioned at 3 defined sites: the proximal hind limb (2 mm beyond the distal suture at the femoral artery excision site), the mid-hind limb (2 mm superior to the mid-knee joint line), and the distal hind limb (the widest muscle portion of the below-knee tissue region).

2.2.4 Histological Analysis: Ischemic Injury Mapping

Five-µm-thick full cross sections of hind limbs were stained for hematoxylin and eosin. Entire cross sections were digitally scanned using a Leica Aperio AT2 Digital Pathology Slide Scanner (Leica Biosystems, Germany) with 40× objective engaged. Skeletal muscle injury areas were identified for all muscles, categorized as either injury-necrosis or injury-regeneration, based on disrupted myofibers devoid of nuclei or regenerated myofibers with
centralized nuclei, respectively (Folker et al., 2013; Hall-Craggs, 1974; Harris et al., 1982). Areas were quantified using ImageJ (National Institutes of Health) and Aperio ImageScope (version 12.3.2.8013; Leica Biosystems). Maps were created based on the locations and the size (area) of the injured territory within a given section (Figure 2.2). Areas were averaged from 9 C57BL/6J mice at day 10 and 5 C57BL/6J mice at day 28. A validation cohort of 5 C57BL/6N mice harvested at day 10 was similarly analyzed. A second validation cohort of 32 inbred C57BL/6J male mice also subjected femoral artery excision was analyzed, evaluating injury maps specifically in the tibialis anterior and distal gastrocnemius muscles.

2.2.5 Histological Analysis: Angiogenesis Mapping

The skeletal muscle vasculature was evaluated in sections near-adjacent to those assessed above by immunostaining for CD (cluster of differentiation) 31-positive endothelial cells. Five-micrometer sections were subjected to antigen retrieval (sodium citrate 0.01 M, pH 6.0 [Sigma], and heating with pressure in a 2100 Retriever device [Prestige Medical]) and immunostained using rat monoclonal anti-mouse CD31 antibody (1:20, Clone SZ31; Dianova) and, in some instances, with rabbit monoclonal anti-mouse Ki-67 antibody (1:400; Abcam). Bound primary antibody was detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:100; Thermo Fisher) and Alexa Fluor-594 conjugated goat anti-rabbit IgG (1:100; Thermo Fisher). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech; 0100-20). Fluorescence imaging was undertaken by widefield microscopy (Olympus BX-51) using the Northern Eclipse image acquisition and analysis software (EMPIX Imaging, Inc) and the Leica Aperio VERSA Digital Pathology Slide Scanner (Leica Biosystems).

Capillary densities for all muscles were quantified in 5 equally spaced high-powered fields per muscle territory, using ImageJ (National Institutes of Health) or Aperio ImageScope. Capillary densities were quantified within the injury zone, border zone, and uninjured zone. The border zone was defined as a single high-powered field of view (225-µm wide) of myofibers with peripheral nuclei, directly adjacent to the myofiber zone with central nuclei. Angiogenesis was deemed to have occurred if the capillary content was statistically
significantly greater than that in the matched contralateral control muscle territory. Angiogenesis maps were generated based on this assessment.

2.2.6 Systematic Publication Review

A search of all publications related to C57BL/6 mouse hind limb ischemia was performed using Medline, Embase, BIOSIS, and Web of Science databases from their respective inceptions (1946, 1947, 1926, and 1900) to July 25, 2019. The initial search strategy was designed to be as inclusive as possible and used the following Medical Subject Headings: mice, ischemia, and hind limb. Subsequently applied exclusion criteria were as follows: abstract-only publications, non-English language publications, review articles, letters and commentaries, studies evaluating collaterogenesis or arteriogenesis rather than capillary-level angiogenesis, mouse models not using the C57BL/6 genetic background, ischemia induced by a surgical technique other than femoral artery excision, and studies that evaluated end points other than skeletal muscle angiogenesis. Non-English articles, review articles, and arteriogenesis articles were identified and excluded based on manuscript title and abstract review. The remaining exclusions were based on a full review of the manuscript and its methods. The final studies included English language peer-reviewed full manuscripts, with histological evaluations of angiogenesis in ischemic skeletal muscle following surgical excision of the femoral artery in C57BL/6 mice. The latter included mice receiving treatment interventions and transgenic mice backcrossed to a C57BL/6 background, to capture the breadth of the relevant studies.

All authors agreed on included and excluded studies. From each of the 509 publications, 3 investigators (J.J.L., H.Y., and J.-M.A.) extracted descriptive and methodologic data. This entailed (1) the year of publication, (2) the age and sex of the mice studied, (3) the vendor from which the mice were obtained and the C57BL/6 sub-strain, (4) whether flow interruption was confirmed by laser Doppler analyses, (5) the day post femoral artery excision angiogenesis was histologically analyzed, (6) the specific muscle(s) evaluated for angiogenesis, (7) whether histological images were provided for control (no femoral artery excision) and injured (femoral artery excision) muscles, (8) whether central/internalized myofiber nuclei were evident in images of injured muscle from which angiogenesis was evaluated, and (9) the consistency of central nuclei among all images of injured muscles.
There was a 99% agreement (506 of 509 articles) on the extracted data. A fourth investigator (J.G.P.) further evaluated the data, and consensus was achieved.

2.2.7 Statistics

Descriptive data are presented as mean ± SD. Comparative data are presented as mean ± SEM. Normal distributions were confirmed using D’Agostino and Pearson omnibus normality testing. Comparisons were made by 2 tailed t tests or ANOVA with Bonferroni post hoc test. Multiple t test corrections were performed using the false discovery rate approach with Q=5% (Glickman et al., 2014). The 2-stage step-up method of Benjamini et al (Benjamini et al., 2006) was used. Associations between the presence of angiogenesis and histological muscle injury status were tested using χ² analysis (Campbell, 2007). Data were analyzed using Prism 8 (version 8.4.2.679; GraphPad Software), and P<0.05 was considered significant unless otherwise stated.

2.3 Results

2.3.1 Femoral Artery Excision Produces Heterogeneous Ischemic Injury With Variable Consistency

To investigate the consistency and distribution of skeletal muscle injury following a strong vascular insult, 9 C57BL/6J mice were subjected to unilateral femoral artery ligation and excision. In all mice studied, the post procedure ischemic to nonischemic hind limb perfusion ratio, determined by laser speckle contrast imaging, fell to at least 0.3 (0.21±0.05) (Figure 2.1). Decalcified hind limbs were harvested in toto, and full transverse sections across upper, mid, and distal hind limb territories were studied. This encompassed 33 distinct planes of 22 different muscles for a given mouse (Figure 2.2A through 2.2C).
Figure 2.1 Hindlimb perfusion in C57BL/6J mice subjected to right femoral artery excision.

A. Laser speckle contrast images depicting paw perfusion before and on the designated days after femoral artery excision.  B. Graph of hindlimb blood flow before and after femoral artery excision. Data are presented as perfusion ratios of the injured ischemic limb to the non-injured non-ischemic limb, up to 28 days after surgery. Day 0 corresponds to immediately after the surgical procedure (mean ± SEM, n = 15 mice). C. Plot of post-operative perfusion ratios vs. the number of muscles showing injury 10 days after surgery, among mice with a post-operative perfusion ratio that had declined to at least 0.3 (n = 15 mice). No correlation between these indices was detected.
Figure 2.2 Atlas of the hindlimb skeletal muscles of C57BL/6J mice subjected to femoral artery excision.

A-C. Maps depicting the skeletal muscles in the proximal (A), mid (B), and distal mouse hindlimb (C). Adjacent to each map is a corresponding hematoxylin and eosin-stained full cross-section of the hindlimb, 10 days after femoral artery excision. Varying intensities of muscle eosinophilia can be seen; less intense staining is present in territories of ischemic injury. D. High-magnification images, corresponding to the outlined zones within the proximal and distal hindlimb, depicting normal (left) and injured (right) regions of the same muscle sections. The uninjured regions have peripheral myofiber nuclei (arrowheads). The injured/regenerating regions have pale myofibers with centralized nuclei (arrowheads).
Myofibers within each cross section were evaluated, and subregions were categorized as (1) uninjured, (2) injured-regenerated (central nuclei), or (3) injured-necrotic (absent nuclei; Figure 2.2D). This analysis was undertaken on 9 mice harvested 10 days after injury and 5 mice harvested 28 days after injury, corresponding to 297 and 165 muscle territories, respectively.

The cross-sectional areas of uninjured, injured-regenerated, and injured-necrotic muscle zones for each cross-sectional plane for every mouse studied are presented in Tables 2.1 and 2.2. Remarkably, for almost every mouse, there were more muscle territories with no evidence for muscle injury (normal myofiber morphology, no features of necrosis, regeneration, fibrosis, or inflammation) than with injury. On average, 14 of 33 muscle territories (±4.7) at day 10 displayed injury, and 14 of 33 territories (±7.7) on day 28 had injury zones. Interestingly, the inter-mouse variability in injury was not related to differences in perfusion. Below the inclusion threshold perfusion ratio of 0.3, there was no correlation between perfusion ratio and the number of injured muscles (P=0.341).

Using the averaged site-specific data, we next generated an atlas of skeletal muscle injury, comprised of injury maps for the full transverse sections across upper, mid, and distal hind limb territories, 10 and 28 days after injury. Both the site and consistency of injury are depicted (Figure 2.3). In the upper hind limb, 10 days after femoral artery excision, a reproducible injury-regeneration response, defined as present in at least 67% of the mice studied, was found for only the vastus medialis, pectineus, and adductor longus muscles. In the mid-hind limb, muscle injury-regeneration was found but no muscle territory displayed reproducible (≥67% consistency) injury. In the distal hind limb, there was more widespread injury, with reproducible injury-regeneration evident in 8 muscles, namely the tibialis anterior, extensor digitorum longus, peroneus longus, flexor digitorum longus, tibialis posterior, popliteus, soleus, and gastrocnemius (Figure 2.3A). Foci of injury-necrosis could be seen within injury-regeneration zones, most prominently in the distal hind limb (Figure 2.3A).

The injury maps of tissues harvested 28 days after surgery were similar for those of day 10. For the upper and mid-hind limb, consistent injury-regeneration was only evident for
Figure 2.3 Hindlimb injury maps for C57BL/6 mice subjected to femoral artery excision.

A. Maps depicting the sites of muscle injury in proximal (left), mid (middle) and distal (right) hindlimb of C57BL/6J mice, 10 days after femoral artery excision. Areas of injury/regeneration are shaded yellow or green, with the respective consistency of injury denoted by the shade. Dark green zones are those with the most consistent injury/regeneration. Areas of injury/necrosis are shaded orange. Map data are based on n=9 mice. B. Maps depicting the sites and consistency of muscle injury in C57BL/6J mice, 28 days after femoral artery excision. Map data are based on n=5 mice. C. Maps depicting the sites and consistency of muscle injury, 10 days after femoral artery excision in the C57BL/6N sub-strain (Charles River). Map data are based on n=5 mice. D. Graph depicting the extent of muscle injury in each of the proximal, mid, and distal hindlimb for the different cohorts of mice analyzed. Data are mean ± SEM. See Tables 2.1-2.3 for individual data values.
Table 2.1 Distribution and size of injury zones 10 days after hindlimb ischemia in C57BL/6J mice.

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Individual C57BL6/J mouse data depicting the relative areas of uninjured (NML), injured-regenerating (RGN), and injured-necrotic (NEC) muscle in 33 hindlimb skeletal muscle territories, 10 days after femoral artery excision. (Prox=Proximal)
Table 2.2 Distribution and size of injury zones 28 days after hindlimb ischemia in C57BL/6J mice.

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<td>Biceps Femoris Anterior (Prox)</td>
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<td>Mouse 3</td>
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<tr>
<td>Mouse 4</td>
<td>88</td>
</tr>
<tr>
<td>Mouse 5</td>
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<tr>
<td>Average</td>
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</tr>
<tr>
<td>Mid Hindlimb</td>
<td>Percent Area of Uninjured, Injured-Regenerating, and Injured-Necrotic Muscle</td>
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<td></td>
<td>Vastus Lateralis (Mid)</td>
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<td>Biceps Femoris Anterior (Mid)</td>
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<tr>
<td>Average</td>
<td>100%</td>
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<td>Distal Hindlimb</td>
<td>Percent Area of Uninjured, Injured-Regenerating, and Injured-Necrotic Muscle</td>
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<tr>
<td></td>
<td>Popliteus</td>
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<td></td>
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<tr>
<td>Mouse 1</td>
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<td>21%</td>
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<th>Semi-tendinosus (Distal)</th>
<th>Biceps Femoris (Distal)</th>
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<td>Reg</td>
<td>Nec</td>
<td>Nor</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>28</td>
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<td>0</td>
<td>0</td>
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<td>99</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Average</td>
<td>59%</td>
<td>41%</td>
<td>0%</td>
<td>6%</td>
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</table>

Individual C57BL6/J mouse data depicting the relative areas of uninjured (NML), injured-regenerating (RGN), and injured-necrotic (NEC) muscle in 33 hindlimb skeletal muscle territories, 28 days after femoral artery excision. (Prox=Proximal)
Table 2.3 Distribution and size of injury zones 10 days after hindlimb ischemia in C57BL/6N mice.

<table>
<thead>
<tr>
<th>Proximal Hindlimb</th>
<th>Percent Area of Uninjured, Injured-Regenerating, and Injured-Necrotic Muscle</th>
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<tbody>
<tr>
<td></td>
<td>Biceps Femoris Anterior (Prox)</td>
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<tr>
<td></td>
<td>NML</td>
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<td>Mouse 1</td>
<td>100</td>
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<td>Mouse 2</td>
<td>100</td>
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<td>Mouse 3</td>
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<td>Mouse 4</td>
<td>100</td>
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<td>Mouse 5</td>
<td>100</td>
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<tr>
<td>Average</td>
<td>100%</td>
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<tr>
<td></td>
<td>Biceps Femoris Posterior (Prox)</td>
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<td>NML</td>
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<td>Mouse 1</td>
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<td>Mouse 5</td>
<td>100</td>
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<tr>
<td>Average</td>
<td>100%</td>
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<tr>
<td>Mid Hindlimb</td>
<td>Vastus Lateralis (Mid)</td>
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<td></td>
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<td>Biceps Femoris Anterior (Mid)</td>
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<td>100</td>
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<tr>
<td>Mouse 5</td>
<td>100</td>
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<tr>
<td>Average</td>
<td>100%</td>
</tr>
<tr>
<td>Distal Hindlimb</td>
<td>Percent Area of Uninjured, Injured-Regenerating, and Injured-Necrotic Muscle</td>
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<td>Mouse 1</td>
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<td>100</td>
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<tr>
<td>Mouse 5</td>
<td>0</td>
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<tr>
<td>Total Avg.</td>
<td>23%</td>
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<th>Semi-tendinosus (Distal)</th>
<th>Biceps Femoris (Distal)</th>
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<td>RGN</td>
<td>NEC</td>
<td>NML</td>
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<td>0</td>
<td>0</td>
<td>79</td>
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<td>22</td>
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<tr>
<td>Mouse 5</td>
<td>10</td>
<td>76</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Total Avg.</td>
<td>44%</td>
<td>46%</td>
<td>8%</td>
<td>27%</td>
</tr>
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</table>

Individual C57BL6/N mouse data depicting the relative areas of uninjured (NML), injured-regenerating (RGN), and injured-necrotic (NEC) muscle in 33 hindlimb skeletal muscle territories, 10 days after femoral artery excision. (Prox=Proximal)
the vastus medialis muscle. In contrast, in the distal hind limb, the 8 muscles consistently showing injury-regeneration on day 10 also consistently displayed injury-regeneration on day 28. Necrotic foci were also seen at day 28, but these zones were less prominent than on day 10.

The injury zones in the distal hind limb occupied all or all but the outer muscle edges of a given muscle, at both 10 and 28 days after femoral artery excision. The exception to this was the gastrocnemius muscle, where much of the gastrocnemius muscle remained uninjured and only deeper regions showed injury-regeneration (Figure 2.3A and 2.3B).

2.3.2 Ischemic Injury Profiles in Validation Cohorts

To determine whether the foregoing injury profiles held with C57BL/6 mice from a different vendor, we undertook the same assessment on 5 mice obtained from Charles River Laboratories. These mice are a sub-strain of C57BL/6 mice (C57BL/6N) that have genomic and phenotype differences with C57BL/6J mice (Simon et al., 2013). Interestingly, the 10-day injury response to femoral artery excision in C57BL/6N was highly similar to that of C57BL/6J mice, in terms of both the site and consistency of muscle injury (Figure 2.3C). The distal hind limb muscles were again the most consistently injured, with the distal anterior hind limb muscles, in particular, almost uniformly injured. We also established that, for a given hind limb zone, the overall proportion of muscle territories displaying injury was no different among C57BL/6J mice harvested at 10 days, C57BL/6N mice harvested at 10 days, and C57BL/6J mice harvested at 28 days (P=0.648; Figure 2.3D).

We also assessed a second validation cohort of 32 inbred C57BL/6J male mice, examining the responses specifically in the tibialis anterior muscle and the gastrocnemius muscle, 14 (n=21) and 28 (n=11) days after femoral artery excision. Both muscles displayed injury in 30 of 32 mice. However, the average relative muscle area occupied by injury-regenerated myofibers in the tibialis anterior muscle was 93.8±4.3%, whereas in the gastrocnemius muscle, this was only 66.0±5.9% (P<0.0001).

Together, these quantitative mapping data reveal that in C57BL/6 mice subjected to femoral artery excision, most muscles display either no features of injury or are partially
and inconsistently injured. Consistent and relatively widespread injury can, however, be found in the distal anterior hind limb.

2.3.3 Angiogenesis Is Regional and Observed in Zones of Injured-Regenerating Muscle

We next quantified angiogenesis in the hind limb muscles of the mice subjected to femoral artery excision. Angiogenesis was defined as a statistically significant increase in capillary density, based on CD31 immunostaining, relative to the matched site of the contralateral hind limb. This assessment was made in regions of uninjured muscle, injured-regenerated muscle, and injured-necrotic muscle.

As depicted in Figure 2.4, angiogenesis was evident within injured-regenerating skeletal muscle territories. On day 10, all but 1 of 25 territories that displayed injury, across the 3 hind limb planes, showed an angiogenesis response. On day 28, 20 of 26 injury zones had statistical evidence for angiogenesis (Figure 2.4B). The average 28-day capillary density in the injured-regenerating zones was found to be less than that on day 10 (1362/mm$^2$ versus 1034/mm$^2$; $P<0.0001$), suggesting pruning of the neocapillaries (Figure 2.4D). Notably, there was no significant increase in capillary density in any of the non-injured muscle territories on either day 10 or 28. Also, in most of the injured-necrotic territories, there was a statistically significant decrease in capillary density, relative to the contralateral muscle territory.

The finding of angiogenesis in injured-regenerating zones but not in uninjured zones or injured-necrotic zones was also observed in the C57BL/6N validation cohort (Figure 2.4).
Figure 2.4 Hindlimb angiogenesis maps for C57BL/6 mice subjected to femoral artery excision.

A. Maps depicting the sites of angiogenesis and capillary loss in proximal (left), mid (middle) and distal (right) hindlimb of C57BL/6J mice, 10 days after femoral artery excision. Angiogenesis and capillary loss were determined based on a statistically significant increase or decrease in capillary density, relative to the corresponding region in the contralateral control muscle, as determined by immunostaining for CD31. Injured/regenerating and injured/necrotic zones are overlaid in dashed lines. Dashed lines surrounding unshaded areas correspond to territories of muscle injury in which a statistically significant increase in capillary density was not found. Map data are based on n=9 mice.

B. Maps depicting the sites of angiogenesis and capillary loss in the hindlimb in C57BL/6J mice, 28 days after femoral artery excision. n = 5 mice.

C. Maps depicting the sites of angiogenesis and capillary loss in the hindlimb in C57BL/6N mice, 10 days after femoral artery excision. Map data are based on n=5 mice.

D. Graph of capillary densities in histologically defined muscle zones for the different cohorts of mice analyzed (mean ± SEM).
2.3.4 Angiogenesis Is Exclusive to Injured-Regenerating Muscle Zones

Because much of the hind limb was not injured after femoral artery excision, we considered the possibility that increases in capillaries in at least parts of the uninjured territories could be missed. For example, angiogenesis might proceed in an injury border zone, recognizing that such a site could be ischemic but without overt muscle injury. To test this possibility, we quantified the capillary density in a 225-µm zone directly adjacent to those injury-regenerated regions around which a border zone could be delineated. As depicted in Figure 2.5, none of 14 border zones were found to have quantitative evidence for angiogenesis. When analyzed together with injured-regenerated zones, injured-necrotic zones, and uninjured zones, contingency analysis revealed an unequivocal and exclusive relationship between angiogenesis and injured-regenerated muscle zones (P=0.0001; Figure 2.5A).

This exclusivity was particularly apparent in relatively large muscles where both injured and uninjured territories were present in similar proportions, such as the gastrocnemius muscle. As depicted in Figure 2.5B, there is a clear demarcation of the edge of angiogenesis zone, and this edge is found precisely at the edge of injury-regeneration zone, that is, the transition from central to peripheral myofiber nuclei. This abrupt spatial transition in angiogenesis was confirmed by quantifying capillary densities of the respective adjacent zones (Figure 2.5C). As well, co-labeling with CD31 and Ki-67 showed no evidence of proliferating endothelial cells in the border zone, despite its abundance in the injury-regenerated zone (Figure 2.5D).
Figure 2.5 Angiogenesis following femoral artery excision occurs exclusively in injured/regenerating muscle zones.

A. Contingency table relating the presence of angiogenesis with histologically-defined muscle zones. Data from a total of 87 zones from nine C57BL/6J mice subjected to femoral artery excision and harvested 10 days later are depicted.  p<0.0001.  B. Micrographs of gastrocnemius muscle sections from of a C57BL/6J mouse subjected to hindlimb ischemia and harvested 10 days later, depicting the transition from injured to non-injured muscle. The upper panel illustrates capillary content, based on immunostaining for CD31 (green) with DAPI (4′,6-diamidino-2-phenylindole) nuclear counterstain (blue). The lower panel is a near-adjacent section stained with hematoxylin and eosin, illustrating the abrupt transition from centralized to peripheral myofiber nuclei.  C. Capillary densities in defined muscle zones of the injured gastrocnemius muscle. *p<0.0001.  D. Fluorescence micrographs of regions in the gastrocnemius muscle 10 days after surgery, immunostained for CD31 (green) and Ki-67 (pink). Nuclei are visualized with DAPI.
2.3.5 Systematic Review: Widely Diverse Muscle Regions Selected for Angiogenesis Assessment

The foregoing data revealed that (1) there is a tight and exclusive linkage between skeletal muscle injury-regeneration and skeletal muscle angiogenesis and (2) only a subset of hind limb muscles consistently undergo injury and regeneration after femoral artery excision. Given these results, we next determined the extent to which published mouse hind limb angiogenesis studies undertook a strategy concordant with the findings. Multi-database structured searching yielded 5147 unique articles from a total of 11,886 article titles generated from the Medical Subject Headings key word search of 4 databases (Figure 2.6). Application of exclusion criteria identified 509 articles, all of which studied angiogenesis in hind limb skeletal muscle following femoral artery excision in C57BL/6 mice.

The earliest of these 509 studies was the 1998 report by Couffinhal et al (Couffinhal et al., 1998) that introduced the mouse hind limb femoral artery occlusion model, providing a measure of internal validity to the search strategy. As noted, all studies undertook a femoral artery excision procedure, and most studies used male mice (Figure 2.7A). Most (88%) studies performed perfusion analyses using a laser Doppler technique. As per the selection criteria, all studies used C57BL/6 mice, and 35% were reported as being obtained from either The Jackson Laboratory or Charles River Laboratories (Figure 2.7B). The median age of mice was 10 weeks (interquartile range, 8–12 weeks). Most mice were histologically analyzed for angiogenesis between 20 and 29 days after femoral artery excision (Figure 2.7C).

The specific skeletal muscles histologically analyzed for angiogenesis are depicted in Figure 2.8. Over half of all manuscripts evaluated angiogenesis in the gastrocnemius muscle (194 of 509, 38%) or the adductor muscle bundle (29%). In the current study, these muscles were inconsistently injured and, when injured, the damage was localized. In contrast, the tibialis anterior and extensor digitorum longus muscles, found in the mapping data to be consistently injured and angiogenic, were an analysis site in only 7% of manuscripts. Also notable was that 20% of studies did not specify which muscles were analyzed for angiogenesis.
Figure 2.6 Systematic literature search strategy and manuscript yield.

Flowchart indicating MeSH (Medical Subject Headings) search terms, exclusions, and resulting yield of studies included for analysis.
To control for any bias that might be introduced by there being multiple studies from the same research group, we repeated the analysis using only the most recently published article from those with the same senior author. This entailed 283 author-unique articles. The distribution of specific muscles analyzed was not demonstrably altered by this author-level adjustment (Figure 2.8B).

2.3.6 Systematic Review: Dissociation Between Zones of Injury and Zones of Angiogenesis Analysis

We next ascertained whether angiogenesis quantification was undertaken at sites of injured-regenerated muscle zones. For this, we evaluated the manuscript figures and ascertained whether central nuclei were evident in the histology images of post femoral artery excision muscle. Most of the 509 studies (94%) displayed hind limb histology images in the main article or the supplemental material. However, unequivocal evidence for central nuclei in at least one of the postinjury hind limb images was present in only 40% of manuscripts and in 43% of those showing muscle histology. The remaining majority of images showed myofibers with exclusively peripheral nuclei or images where the nuclear location could not be interpreted, either because of suboptimal image quality or the lack of a nuclear stain. Furthermore, in only 15% of all articles, was there a consistent depiction of central nuclei in all of the postinjury hind limb images shown (Figure 2.9A). Author-level adjustment did not impact these findings (Figure 2.9A).

We also ascertained whether the specific muscle analyzed for angiogenesis was related to the likelihood of there being centralized nuclei in images. This proved to be the case. In those minority of manuscripts that studied distal anterior hind limb muscles, the probability of there being centralized nuclei was high (odds ratio, 5.6 [95% CI, 1.3–25.4]; Figure 2.9B). In contrast, those manuscripts that studied angiogenesis in either gastrocnemius or adductor muscles were likely not to show centralized nuclei (odds ratio, 0.2 [95% CI, 0.04–0.8]; Figure 2.9B).

Collectively, this body of curated manuscript data points to a common discordance between the site of angiogenesis analysis and the probable site of angiogenesis.
Figure 2.7 Methodological metrics in published literature evaluating angiogenesis following hindlimb ischemia.

A. Graph showing the distribution of manuscripts based on the sex of mice used for angiogenesis assessment. B. Graph showing the distribution of manuscripts based on the vendor from which mice were procured. C. Distribution of manuscripts according to the time points at which angiogenesis analysis following femoral artery excision was undertaken.
Figure 2.8 Analysis of muscles analyzed in the published literature evaluating post-ischemia angiogenesis.

A. Graph showing the prevalence of the specific muscles used for histological angiogenesis evaluation and quantitation, among all manuscripts analyzed (n=509 manuscripts). B. Graph showing the prevalence of the specific muscles used for histological angiogenesis evaluation, after author-level adjustment to account for multiple manuscripts from the same research group (n=283 manuscripts).
Figure 2.9 Analysis of injured muscle zones studied in the published literature.

A. Pie-charts showing the distribution of manuscripts based on the state of the skeletal myofibers evaluated for post-injury angiogenesis, as depicted in the representative histology images. Chart on the left is for all 509 manuscripts; chart on the right is for the 283 unique senior author manuscripts. B. Depiction of the probability of there being centralized nuclei in histological images of tissues from mice subjected to femoral artery excision, depending on the specific muscles analyzed. Data on right indicate the odds ratio (OR) and 95% confidence intervals for the presence of central nuclei in the images.
2.4 Discussion

We have undertaken a unique, hybrid investigation of the widely used mouse model of ischemia-induced hind limb angiogenesis. This entailed generating a comprehensive atlas of muscle injury and angiogenesis and combining this with a systematic review of the published literature. We used the mapping data to inform a methodological analysis of this large body of manuscripts. In so doing, we have identified elemental vulnerabilities in the mouse hind limb ischemia model that could hinder data reliability and, potentially, clinical translation.

Systematic mapping of the muscle injury and angiogenesis landscapes was enabled by evaluating full transverse sections across the decalcified hind limb at upper, mid, and distal levels. This assessment revealed that, despite pronounced loss of perfusion induced by femoral artery excision, skeletal muscle injury was often inconsistent and always regionalized. Regional variability was evident among different muscles, but also within a given muscle, including within the same cross-sectional plane. We also established that all regions of muscle throughout the injured hind limb could be classified as being in 1 of 3 states—uninjured, necrotic, or regenerating. This proved to be critically important for the study of angiogenesis. Uninjured muscle, which constituted most of the hind limb, showed no angiogenesis whatsoever. Necrotic muscle, found as small internal zones in select muscles, displayed a reduction in capillary count. Postinjury regenerated muscle, and only those zones, displayed angiogenesis. These conclusions were based on the assessment of 691 hind limb muscle territories from 51 C57BL/6 mice. Moreover, the angiogenesis-regenerated muscle relationship existed for tissues harvested both 10 and 28 days after femoral artery excision and in mice obtained from 2 different vendors.

Our finding of an irrevocable linkage between angiogenesis and postischemic regenerating muscle has important implications for the assessment of angiogenesis. Because the majority of the hind limb muscle was not regenerating, there is a risk of harvesting tissue and evaluating angiogenesis in a territory of muscle that will not have it. As well, given the inconsistency and spatial heterogeneity of angiogenesis, there is a risk of inadvertently mismatching tissue regions harvested for control and treatment interventions. Importantly, our mapping data provide 2 strategies with which to mitigate these risks. First, central
myofiber nuclei, which denote regenerating skeletal muscle in injured mice (Folker et al., 2013), provide a natural strategy for identifying a suitable zone for angiogenesis analysis. Second, the hind limb atlas established that the distal anterior muscles of C57BL/6 mice have a high likelihood of injury and angiogenesis, affording a strategy for site reliability.

The systematic manuscript review revealed that the identified data acquisition vulnerabilities in the mouse model are vulnerabilities that can play out in published studies. We reviewed 509 manuscripts that studied hind limb angiogenesis in C57BL/6 mice subjected to femoral artery excision, conditions similar to those for generating the injury and angiogenesis maps. Remarkably, only 15% of manuscripts consistently assessed angiogenesis in regions of muscle that displayed regeneration, as indicated by the presence of central nuclei in the corresponding images. Furthermore, only 7% of the analyzed manuscripts evaluated angiogenesis in muscles within the distal anterior hind limb, that is, those with the greatest likelihood of being injured and undergoing angiogenesis. Although we cannot definitively link the sites analyzed with the capillary content results, the disparities with the mapping data highlight the potential for reproducibility challenges and possibly inaccurate conclusions. For example, if the site used to assess a test intervention was a regenerating zone but the site used to assess the control intervention was not, this could lead to reporting a pro-angiogenesis effect of the intervention where one actually does not exist (type I error). Conversely, if each territory used to assess the control and test interventions were not in fact subjected to ischemic damage, an angiogenesis effect could be missed (type II error). These are theoretical scenarios that arise from our data analysis, but also tangible risks given the observed heterogeneity within the vast muscle landscape and the sampling inherent in the microscopic assessment of angiogenesis.

The systematic review established that the most commonly used muscles for angiogenesis analysis have been the gastrocnemius muscle and adductor muscle groups. This is not surprising as these are 2 large and easily accessible hind limb muscle tissues (Heil et al., 2006; Limbourg et al., 2009; Scholz et al., 2002). It is notable, therefore, that these muscles were also among those that underwent variable and focal injury and angiogenesis. Why the gastrocnemius muscle—a distal posterior hind limb muscle—was less reliably impacted than the distal anterior muscles is unknown but could reflect collateral network differences
Evaluating the most distal aspect of the gastrocnemius muscle has been recommended and may lessen the risk (Limbourg et al., 2009). We propose that an analysis strategy that is (1) oriented around centralized nuclei and (2) takes into account any residual zones of necrosis would be important to optimize the value of this muscle. In fact, our data imply that this strategy should hold for any muscle studied, including both oxidative and glycolytic.

Approaches to enhancing the reliability of angiogenesis assessments have recently emerged. A seminal methods report on mouse hind limb ischemia has been published (Limbourg et al., 2009), as well as technical variations (Hellingman et al., 2010). An expert-guided consensus document on the use and interpretation of several angiogenesis assays has also been published (Nowak-Sliwinska et al., 2018). To our knowledge, the current study is the first to map all injury and all angiogenesis zones in the entire hind limb and the first to employ a systematic manuscript review algorithm in the basic angiogenesis field. Systematic reviews are uncommon for basic and preclinical research topics, although recognition of their value is emerging (Ramirez et al., 2017; Suen et al., 2019; Zwetsloot et al., 2016). Our results add to a growing appreciation of the need for quality assurance in preclinical studies, and they provide a precision-based framework for optimizing the analysis of the injured mouse hind limb.

We restricted our de novo mapping study to young and otherwise healthy mice. Older mice can be expected to show more injury, and the angiogenesis responses could be different (Frontini et al., 2011; Lotﬁ et al., 2013; Nowak-Sliwinska et al., 2018). We also only mapped injury and angiogenesis in male mice, to align the data with existing literature. Importantly, our systematic manuscript review quantified the experimental sex bias in the field, establishing that only 10% of manuscripts assessed female mice. Reduced flow recovery has been reported in female mice (Peng et al., 2011), and it will be important to systematically assess for sex differences in the angiogenesis landscapes. We note also that arteriogenesis responses and the state of collateral vessels are important determinants of the response to ischemia that we have not investigated (Faber et al., 2014; Hellingman et al., 2010; Zbinden et al., 2007). Like angiogenesis, these vascular responses are also challenging to quantify, and we propose that the current hybrid mapping-evaluative study
of angiogenesis could inform a similar evaluation of the complex feeder vessel response to ischemia.

We also limited the injury and angiogenesis mapping to two commonly used C57BL/6 mouse sub-strains. Other mouse strains can be expected to have different responses, as will mice subjected to superimposed metabolic and inflammatory challenges (Helisch et al., 2006; S. M. Marques et al., 2011). The latter may better reflect the human peripheral artery disease scenario. That said, we propose that intra-mouse delineation of skeletal muscle territories as either normal, necrotic, or regenerating, and focusing the angiogenesis assessment accordingly, could be a quality assurance tool that holds across mouse strains, vascular risks, the metabolic phenotype of specific muscles, and modes of inducing ischemia.

Finally, it will be important to ascertain whether the identified linkage between the state of the skeletal muscle and angiogenesis also holds for humans with peripheral artery disease. In this regard, we recently identified central skeletal muscle nuclei and evidence for angiogenesis in tibialis anterior muscle samples in patients with critical limb ischemia (Chevalier et al., 2020).

In summary, the mouse hind limb ischemia model is an investigative mainstay for exploring therapeutic innovations. However, we have established that evaluating angiogenesis in this model carries substantial data risks. Developing quality assurance parameters, based, in part, on the mapping and review data herein, could augment data reliability and potentially help translate mouse hind limb ischemia studies to patient care.

2.5 References


Ischemic Muscle Produces a Flawed Microcirculation. *Circ Res*, 120(9), 1453-1465. doi:10.1161/CIRCRESAHA.116.310535


limb loss by overexpression of vascular endothelial growth factor 165 but not of fibroblast growth factor-2. Circ Res, 90(9), 966-973. doi:10.1161/01.res.0000019540.41697.60


ischemia. *Am J Physiol Heart Circ Physiol, 300*(6), H2027-2034. doi:10.1152/ajpheart.00004.2011


CHAPTER 3

3 Myh11+ Progenitor Cells Generate Skeletal Myofibers Following Ischemic Injury in the Mouse Hindlimb

3.1 Introduction

Peripheral artery disease (PAD) is a widespread clinical problem that can damage skeletal muscle and lead to limb loss (Farber, 2018; Song et al., 2019). Unlike cardiac muscle, skeletal muscle can regenerate, owing to muscle resident stem cells, termed satellite cells (Ryall, 2013; Wagers et al., 2005). This raises prospects for therapeutically targeting skeletal muscle regeneration in PAD. However, satellite cells may not be the sole muscle stem cell (N. Liu et al., 2017; Wagers et al., 2005), making it important to know if other progenitors participate in regeneration post-ischemia.

Pericytes are microvascular mural cells that in experimental settings display stem cell attributes (Crisan et al., 2008; Nwadozi et al., 2020; Ray et al., 2020). However, whether endogenous pericytes give rise to skeletal muscle fibers, particularly in adults, is unclear. One study reported pericyte-based myogenesis in the early post-natal period; however, during adult myogenesis any pericyte lineage signal found was in only part of a myofiber segment and there was no increase in signal following toxin injury (Dellavalle et al., 2011). A subsequent study reported similar results in the adult mouse using different lineage tracing markers and showed that pericytes did not contribute to adult myogenesis at all, either at baseline or with toxin-induced regeneration (Guimaraes-Camboa et al., 2017). Thus, these studies highlight the lack of direct in vivo evidence for pericyte-derived myogenesis in the adult and even proof-of-concept for a perivascular cell-driven regeneration is uncertain.

In the clinical context of PAD and its associated ischemic injury of skeletal muscles, the question of pericyte-based myogenesis is unanswered. Animal models used to test pericyte contributions to skeletal muscle regeneration have been limited to myotoxin or barium chloride-induced muscle injury (Hardy et al., 2016). When evaluating pericyte-based myogenesis in PAD, however, ischemia may be a more clinically relevant injury.
(Couffinhal et al., 1998; Nowak-Sliwinska et al., 2018). A mouse model of femoral artery excision, established by Couffinhal et al. (Couffinhal et al., 1998) in 1998, is one of the most widely used animal models of ischemia-induced angiogenesis and skeletal muscle regeneration. An ischemia model of skeletal muscle injury would thus be an important context for investigating the potential biology of pericyte reprogramming (Ryall, 2013).

In addition to the differences between various animal models of injury, endogenous factors that may regulate the transition of perivascular cells to skeletal myofibers are poorly understood. Indeed, a limitation of pericyte-derived cell therapy is that the pericytes in these studies are purified, cultured and treated prior to re-administration into injured tissues (Crisan et al., 2009; Crisan et al., 2008). The innate multipotent potential of pericytes cannot be answered with this approach. Likewise, identification of endogenous factors that may be important to the pericyte-myofiber transition cannot be ascertained. Metabolic pathways have been implicated in pericyte reprogramming (Nwadozi et al., 2020). One class of regulators at the intersection of metabolism and cellular reprogramming are sirtuin histone deacetylases (Hsu et al., 2018; Y. L. Lee et al., 2012; Sharma et al., 2013). Of these, Sirt6 has been shown to influence glucose metabolism (Mostoslavsky et al., 2006; Raj et al., 2020) and play a critical role in cellular reprogramming and differentiation of multipotent cells (Etchegaray et al., 2015; Sharma et al., 2013; P. Xu et al., 2019). Sirtuins are of further interest because of their susceptibilities to pharmacological activators and inhibitors.

In this study, I sought to determine if cells of a smooth muscle myosin heavy chain (Myh11)-expressing lineage could give rise to regenerated skeletal myofibers in adult mice subjected to hindlimb ischemia. Lineage tracing studies have shown that Myh11-Cre reporter mice mark vascular smooth muscle cells and most pericytes in skeletal muscles (Hess et al., 2019). I therefore exploited this system of inducible permanent fluorescent labelling of vascular mural cells. Furthermore, I investigated the impact of Sirt6 knockout in (Myh-11)-expressing cells on their multipotent potential. Our results provide evidence for mural cell-based skeletal muscle regeneration after ischemic insult and identify Sirt6 as a regulator of this multipotent behaviour.
3.2 Materials and Methods

3.2.1 Myh11-CreERT2 R26-mT/mG Fluorescent Labelled Reporter Mice

Experiments were conducted in accordance with the University of Western Ontario Animal Care and Use Subcommittee, which follows the policies set out by the Canadian Council on Animal Care. This study was also conducted in accordance with the Animal Research: Reporting In Vivo Experiments guidelines for transparent reporting of animal research (Kilkenny et al., 2010) and the 2018 consensus guidelines for the use and interpretation of angiogenesis assays (Nowak-Sliwinska et al., 2018). We bred mice harboring a Myh11-CreERT2 allele into R26-mT/mG mice (Jackson strain: B6.129(Cg)-Gt(Rosa26)26Sortm4(ACTB-TdTomato,-EGFP)Luo/J), both of C57BL/6 genetic background. The mice constitutively express a membrane-targeted TdTomato protein from the Rosa26 locus, but when Cre is activated the red fluorescence is replaced by that of membrane-targeted eGFP. Eight-week-old mice received tamoxifen for 5 days. Four weeks after the last dose of tamoxifen the mice were subjected to right femoral artery ligation and excision, and muscles were evaluated 10 days later. Mice were housed in clear, plastic cages containing standard bedding and maintained on a 12-hour light and dark cycle at 23°C and 50% relative humidity. Mice were fed water and normal mouse chow diet (Diet 2018; Harlan Teklad, Madison, WI), which were available ad libitum.

3.2.2 Generation of a Vascular Mural Cell Specific Sirt6-Deficient Fluorescent Reporter Mouse Model

Male mice carrying the Myh11-CreERT2 allele as well as R26-mT/mG alleles were crossbred with Sirt6\textsuperscript{flox/flox} females of FVB background (The Jackson Laboratory, Sirt6tm1.1Cxd/J, 017334) to produce male offspring that were heterozygous for floxed Sirt6 (Sirt6\textsuperscript{flox/wt}) with Cre and mT/mG expression. These offspring were back-crossed with C57BL/6 mice for over 10 generations to produce Myh11-CreERT2, R26-mT/mG, Sirt6\textsuperscript{flox/wt} mice of C57BL/6 genetic background. Finally, heterozygous offspring were mated together to generate homozygous floxed mice as well as littermate WT controls. To identify Sirt6\textsuperscript{flox/flox} mice, Sirt6 WT and floxed alleles are amplified using primers P1: 5’
AGT GAG GGG CTA ATG GGA AC 3’ and P2: 5’ AAC CCA CCT CTC TCC CCT AA 3’.

Eight-week-old mice received tamoxifen for 5 days. Four weeks after the last dose of tamoxifen the mice were subjected to right femoral artery ligation and excision, and muscles were evaluated 10 days later. Mice were housed in clear, plastic cages containing standard bedding and maintained on a 12-hour light and dark cycle at 23°C and 50% relative humidity. Mice were fed water and normal mouse chow diet (Diet 2018; Harlan Teklad, Madison, WI), which were available ad libitum.

### 3.2.3 Mouse Model of Hind Limb Ischemia

Hind limb ischemia was induced by ligating the right femoral artery above and below the proximal caudal femoral artery (Couffinhal et al., 1998; Kochi et al., 2013), also known as profunda femoris branch, using 6-0 silk sutures and excising the intervening 2- to 3-mm portion of artery (Frontini et al., 2011; Limbourg et al., 2009). Bulk blood flow to the distal hind limb was assessed using laser speckle contrast imaging (moorFLPI-2; Moore Instruments, United Kingdom) (J. J. Lee et al., 2020). Relative limb perfusion was measured pre- and post-ischemia using regions of interest confined to the plantar soles, as reported previously (Limbourg et al., 2009). Mice were lightly anesthetized using isofluorane (1.3%–1.5% mixed with 100% oxygen at a rate of 0.8 L/min) and placed on a heating pad, covered by a black mat provided by the manufacturer, to maintain a body temperature of 36.5±0.5°C. The mice were acclimatized to the anesthetic and heating pad for 5 to 7 minutes before imaging and flow data collected according to manufacturer instructions. Image settings were set to Temporal Measure Mode with a Temporal Filter of 250 frames. Postprocessing of images and data extraction were performed using moorFLPI Review V5.0 software (Moore Instruments). Bulk blood flow in the ischemic limb was calculated as the ratio of perfusion in the ischemic (right) limb versus the nonischemic (left) limb. Mice with postoperative day 0 perfusion ratios that did not decline to 30% of baseline or lower were excluded, in line with currently accepted standards (J. J. Lee et al., 2020; Limbourg et al., 2009) and allowing for the higher spatial resolution of laser speckle contrast imaging compared with laser Doppler imaging. Live animal imaging of the EDL muscle was performed by intravital microscopy and all hind limb muscles were harvested.
after 10 days, a time point in which capillary regeneration is known to be robust (Arpino et al., 2017).

### 3.2.4 Intravital Microscopy

Skeletal myofibers and their associated microvascular network within a 50-μm-deep zone across the entire mouse extensor digitorum longus (EDL) surface was assessed by live fluorescent intravital microscopy. Briefly, mice were anesthetized using isofluorane (1.3%–1.5% mixed with 100% oxygen at a rate of 0.8 L/min) and placed on a heating pad to maintain a body temperature of 36.5±0.5°C. To expose the EDL muscle surface, 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. The EDL was covered with an 8×8-mm glass coverslip and positioned face-down on the stage of an inverted fluorescence microscope (Nikon A1R Confocal Laser Scanning System, Nikon, Japan). After a 20-minute stabilization period, fluorescent images of the EDL were acquired using the Galvano scanning mode with lasers at 488, 561, and 640 nm excitation wavelengths as appropriate through the NIS Elements software (Nikon, Japan). Intravascular space was identified by intra-penile injection of AngioSense 680EX fluorescent imaging agent ($7 \times 10^4$ MW; 100 μL; NEV10054EX, PerkinElmer).

### 3.2.5 Histology and Immunostaining

Whole hind limbs were dissected, immersed in 4% paraformaldehyde overnight, and then decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days (Arpino et al., 2017; J. J. Lee et al., 2020). Each hind limb was embedded in paraffin and sectioned at the distal hind limb (the widest muscle portion of the below-knee tissue region). Skeletal muscle injury areas were recognized for all muscles and regenerated myofibers were further distinguished by their centralized nuclei (Folker et al., 2013; Hall-Craggs, 1974; Harris et al., 1982; J. J. Lee et al., 2020).

To comprehensively interrogate for Myh11$^+$ cellular lineage, full transverse 5 μm sections of decalcified hindlimbs were double-immunolabeled for TdTomato (mT) and eGFP (mG) using rabbit polyclonal anti-RFP antibody (1:100; Rockland, 600-401-379-RTU) and goat
polyclonal anti-GFP antibody (1:200; Abcam, Ab5450) respectively. Bound primary antibodies were detected using Alexa Fluor-488 conjugated donkey anti-goat IgG (1:100; Thermo Fisher, A11055) and biotin conjugated donkey anti-rabbit IgG (1:100; Jackson Immuno-Research 711-065-152) along with streptavidin Dylight-549 (1:00; Vector Labs, SA5549). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech; 0100-20). Fluorescence imaging of histological preparations were undertaken by widefield microscopy (Olympus BX-51) using the Northern Eclipse image acquisition and analysis software (EMPIX Imaging, Inc). Structural quantification of myofiber characteristics were performed using ImageJ (National Institutes of Health).

3.2.6 Statistics

Descriptive data are presented as mean ± SD. Comparative data are presented as mean ± SEM. Normal distributions were confirmed using D’Agostino and Pearson omnibus normality testing. Comparisons were made by 2 tailed t tests for normally distributed data. For non-normally distributed data, Mann-Whitney U test was performed. Data were analyzed using Prism 8 (version 8.4.2.679; GraphPad Software), and p < 0.05 was considered significant unless otherwise stated.

3.3 Results

3.3.1 C57BL/6J Mice Harboring Myh11-CreERT2, R26-mT/mG Reporter Transgenes Selectively Label Vascular Smooth Muscle Cells and Pericytes

To investigate the myogenic potential of vascular mural cells in vivo, a lineage tracing system with specific labelling of vascular mural cells was generated using transgenic reporter mice. Specifically, I bred mice harboring a Y-chromosome embedded Myh11-CreERT2 allele into R26-mT/mG mice, both of C57BL/6 genetic background. These transgenic lineage tracing reporter mice constitutively express a membrane-targeted TdTomato protein from the Rosa26 locus, but when Cre is activated in Myh11+ mural cells via tamoxifen induction the red fluorescence is replaced by that of membrane-
Figure 3.1 Generation of a Myh11+ fluorescent mural cell transgenic lineage tracing system and experimental protocol.

A. Schematic of Myh11+ lineage tracing, using Myh11-CreERT2; R26-mT/mG mice and tamoxifen delivery. B. Timeline of experimental interventions.
targeted eGFP. This enabled permanent fluorescence tracking of mural cells in vivo via live animal imaging methods as well as immunohistochemical analyses.

In this study, seven wild type control mice with Sirt6$^{WT/WT}$, Myh11-CreERT2, R26-mT/mG alleles along with seven knockout mice with Sirt6$^{flox/flox}$, Myh11-CreERT2, R26-mT/mG alleles were treated with intraperitoneal injections of tamoxifen for 5 days. Prior to tamoxifen injections, only Td-Tomato-RFP signal was seen throughout the entire hindlimb tissue. Subsequently, after a 4-week washout period, femoral artery ligations and excisions were performed. After 10 days following surgery, live animal intravital fluorescence microscopy was performed. Finally, whole distal hindlimbs were decalcified and sectioned for histological analyses (Figure 3.1). In total, 14 extensor digitorum longus (EDL) muscles were visualized by intravital microscopy and 112 distal hindlimb muscles were histologically analyzed by immunohistochemical methods. Here, images obtained from both live animals via intravital fluorescence microscopy as well as immunohistochemical analyses revealed arterioles and veins surrounded by mG-expressing smooth muscle cells, and capillaries by mG-expressing pericytes, confirming appropriate cell marking by the Myh11 transgene.

3.3.2 Myh11$^+$ Mural Cells Generate Structurally Similar New Skeletal Myofibers Following Femoral Artery Excision

Upon confirmation of mural cell fluorescent labelling and tracking, I next sought to investigate the myogenic potential of vascular mural cells in the in vivo ischemic hindlimb system. This was accomplished via immunostaining for the presence of eGFP and TdTomato-RFP. Again, 10 days following femoral artery excision, whole distal hindlimb tissues were decalcified by emersion in 6.5%, pH 7.0 EDTA solution for an additional 10 days. Then, by visualizing the structurally intact whole distal hindlimb in 5µm cross-sections we were able to conduct unbiased analyses of every distal hindlimb muscle. In total, 112 distal hindlimb muscles were closely examined.

As expected, RFP signal was detected in all tissue and cell types, with the exception of eGFP$^+$ mural cells noted previously. Within uninjured control muscle sections, all myofibers were labelled by RFP and none by GFP. Remarkably, Myh11$^+$-derived green-
fluorescing skeletal myofibers were present in every wild-type control mice studied (n=7). A total of 43 Myh11-derived regenerating myofibers were discovered across these 7 wild-type mice, with one mouse harboring 26 green-fluorescing myofibers. These Myh11\(^+\) lineage myofibers were thus of relative low abundance, with no predilection for a specific muscle. Interestingly, there did not appear to be any overlap of membrane targeted eGFP and Td-Tomato RFP signals within the Myh11-derived regenerating myofibers, suggesting homogeneous lineages of individual myofibers (Figure 3.2).

Further structural analyses were performed via histological methods on all 43 Myh11-derived regenerating myofibers. Again, membrane targeted fluorescence labelling allowed for precise measurements of individual myofibers. The Myh11-derived regenerating myofibers found in wild type mice were always located within 200 µm (87.2 µm ± 50.4 µm) of the outer border of a myofiber bundle, near the perimysium. As well, only regenerated myofibers, denoted by central nuclei, were Myh11\(^+\) cell-derived. Next, an additional 32 regenerated mT-expressing myofibers were analyzed for comparison. Here, both Myh11-derived mG-expressing myofibers and mT-expressing myofibers were of similar size, which were of expected sizes at day 10 post femoral artery excision and smaller than non-injured native myofibers (Figure 3.3). More specifically, mG-expressing myofibers had a cross-sectional area of 574 µm\(^2\) compared to 540 µm\(^2\) in mT-expressing myofibers (p = 0.68). Similarly, mG-expressing myofibers had a cross-sectional diameter of 34.4 µm compared to 35.6 µm in mT-expressing myofibers (p = 0.64).

3.3.3 Myh11\(^+\) Derived Regenerated Myofibers are of Homogeneous Cellular Lineage

Myofibers form by fusion of myoblasts. Thus, green fluorescence in a myofiber could in theory arise from as little as a single Myh11\(^+\) progenitor-derived myoblast. To determine the extent to which a green-fluorescing myofiber was constituted by Myh11\(^+\)-lineage cells, and also validate the findings from cross-sectional immunostaining analyses, the EDL muscle was imaged in live mice using a Nikon A1R confocal laser scanning intravital microscopy system. Again, 10 days after femoral artery excision of the right hindlimb, the distal anterior hindlimb muscles were exposed via surgical reflection of the overlying skin and the tibialis anterior muscle. This enabled stitched volumetric image
Figure 3.2 Fluorescent lineage tracing identifies Myh11\(^+\) mural cells as well as transdifferentiated regenerating myofibers.

Images (BX51, Olympus) of gastrocnemius muscle 10 days after femoral artery excision immunostained for eGFP (abcam, ab5450) and TdTomato (Rockland, 600-401-379-RTU). Bottom Left: Myh11\(^+\) mural cells surrounding arterioles and a venule. Bottom Right: Myh11\(^+\) mural cells surrounding capillaries and a green Myh11\(^+\) cell lineage skeletal myofiber with central nuclei. Scale bars represent 35 \(\mu m\).
Figure 3.3 Structural analyses of Myh11+ mural cell-derived regenerated myofibers in cross-sectional dimensions.

Cross-sectional area of regenerated myofibers and cross-sectional diameter of regenerated myofibers were measured at day 10 post femoral artery excision.
Figure 3.4 Intravital microscopy detects a myofiber of homogenous Myh11+ lineage. Top. Confocal images (488 nm and 561 nm lasers, Galvano scanner) of EDL muscle in live mice showing a homogenously green-fluorescing skeletal myofiber in long axis (1 µm-thick z-slice, pixel resolution 625 nm). Arrows denote cell bodies of pericytes surrounding a microvessel and arrowhead denotes a bridging pericyte with a process along a capillary adjacent to the myofiber. Bottom. Z-plane reconstructed cross sections (55-µm thick) showing Myh11+ cell lineage skeletal myofiber and capillary mural cells.
Figure 3.5 Ultra-structure analysis of regenerated myofiber sarcomere length via high resolution intravital fluorescence microscopy.

Linear depiction of sarcomere lengths in non-Myh11<sup>+</sup> cell-derived regenerated myofibers (red) as compared to Myh11<sup>+</sup> cell-derived regenerated myofibers (green).
acquisitions of the EDL muscle along their entire length and up to 50 µm in depth by z-stacked fluorescent images. The native fluorescence from eGFP and Td-Tomato clearly provided definitive signals that were distinguishable from autofluorescence (Figure 3.4).

As a further internal control of the specificity of the eGFP signal, we were able to confirm that there were abundant microvessels wrapped by cells of Myh11⁺ lineage. In addition, there were discrete homogeneously mG-expressing skeletal myofibers with central nuclei coursing the entire length of the EDL muscle. High resolution images (voxel dimensions: 0.625µm × 0.625 µm × 1 µm) with membrane targeted fluorescence proteins provided the opportunity for further structural analyses of Myh11-derived regenerated myofibers. Their sarcomere length was similar to that of mT-expressing fibers, implying normal ultrastructure (Figure 3.5). The green fluorescence was again homogeneous along the entire imaged myofiber length (up to 3.5 mm), with no evidence for overlapping red fluorescence. The lack of mixed colour signal suggests that mG-expressing myofibers were not a product of Myh11⁺ lineage cells fusing with existing myofibers, or fusing with mT satellite cells or mT myoblasts. Rather, they were distinct myofibers formed *de novo* from Myh11⁺ progenitors, with homologous fusion of Myh11⁺ stem cells or their progeny.

3.3.4 Sirt6 Deficiency in Myh11⁺ Vascular Mural Cells Severely Impair their Myogenic Regenerative Potential

Next, I sought to explore the effects of Myh11⁺-Sirt6 knockout on the multipotent potential of Myh11⁺ vascular mural cells in the ischemic hindlimb. Interestingly, it has been shown that Sirt6 is vital for generation of inducible pluripotent stem cells (iPSCs) from adult fibroblasts (Sharma et al., 2013; P. Xu et al., 2019) as well as differentiation of stem cells during bone development and homeostasis (Sun et al., 2014; P. Zhang et al., 2017), tooth development (Liao et al., 2017), and adipogenesis (Chen et al., 2017). Therefore, we analyzed the distal hindlimb for evidence of Myh11-expressing mural cell reprogramming and transformation in the setting of Myh11⁺-Sirt6 knockout. To achieve this, we generated unique transgenic mice carrying homozygous *Sirt6*<sup>flox/flox</sup> alleles in addition to the established Myh11-CreERT2, R26-mT/mG C57BL/6 background. Upon tamoxifen induced recombination, Myh11-expressing cells undergo deletion of the functional *Sirt6* gene. In total, after femoral artery ligation and excision, 56 distal hindlimb muscles from
7 knockout mice were studied, in comparison to the established group of control wildtype mice.

Compared to wild-type control mice, which display a consistent myofiber regenerative response involving Myh11\(^+\)-derived cellular trans-differentiation, Myh11\(^+\)-Sirt6 knockout mice lacked these Myh11\(^+\)-derived regenerated myofibers (Figure 3.6). More specifically, seven out of seven control mice had at least one hindlimb muscle with Myh11\(^+\)-derived regenerating myofibers, and 5 of 7 mice had more than two muscles that contained Myh11\(^+\)-derived myofibers. In this group, 30% of all distal hindlimb muscles (17 of 56) contained a Myh11\(^+\)-derived regenerating myofiber, and 12 of these 17 muscles were found in the anterior hindlimb. In contrast, only 5 of 7 Myh11\(^+\)-Sirt6 knockout mice had at least one muscle with Myh11\(^+\)-derived regenerating myofibers within the distal hindlimb and only 1 out of 7 mice had two muscles that contained a Myh11\(^+\)-derived regenerating myofiber. In the knockout group, only 11% of all distal hindlimb muscles (6 of 56) contained a Myh11\(^+\)-derived regenerating myofiber (p = 0.02) and only 2 of the 6 muscles were found in the anterior hindlimb. Remarkably, we observed that Sirt6 knockout in Myh11-expressing vascular mural cells impair the myogenic potential \textit{in vivo}, as determined by a lack of Myh11-derived regenerated myofibers in the injured hindlimb.
Figure 3.6 The abundance of Myh11+ mural cell-derived regenerated myofibers in Myh11+-Sirt6 knockout mice are significantly diminished.

The abundance of Myh11+ mural cell-derived regenerated myofibers in Myh11+-Sirt6 knockout mice are severely diminished, as compared to their wild type littermate controls (p = 0.0039).
3.4 Discussion

Cell-based therapy for ischemic skeletal muscle injury has tantalizing therapeutic potential in restoring both structure and function upon muscle damage. Stem cell therapies for treatment of PAD have shown promise in various animal models and human studies (Rigato et al., 2017); however, translating these results into clinical outcomes have been met with several challenges, including effective cell delivery. Recently, vascular mural cells, and more specifically, pericytes, have demonstrated multipotent potential, particularly in mouse models at younger ages (Crisan et al., 2008; Dellavalle et al., 2011; Nwadozi et al., 2020). In this regard, pericyte-derived regeneration of skeletal myofibers in adult tissues, particularly in the setting of ischemia, has yet to be fully explored. In this study, we induced hindlimb ischemia via femoral artery ligation and excision. Upon confirmation of ceased bulk blood flow, followed by subsequent regeneration of skeletal muscle and the microvascular network, we discovered new evidence supportive of Myh11-cell-lineage-derived mural cell generation of new skeletal myofibers, which integrated into the larger muscle architecture as a whole.

Assessing cellular multipotency via reprogramming of somatic adult cells requires the ability to monitor and analyze individual cells within tissues along a time-course. In transgenic animal models, this can be achieved using conditionally inducible fluorescent labelling, which tags cells so that they can be studied microscopically. In our study, vascular mural cells, including the majority of pericytes, were lineage labelled by Myh11 promoter driven Cre expression, which was coupled with a fluorescent transgene driven by the constitutively active ROSA26 promoter. Using this strategy we confirmed previous reports of Myh11+ mural cell labelling of not only vascular smooth muscle cells, but also pericytes throughout the skeletal muscle. This confirmation was performed using two independent microscopy methods – traditional fixed-tissue immunofluorescence microscopy as well as live animal intravital fluorescence microscopy.

Once vascular mural cell labelling by the Myh11-Cre, R26-mT/mG system was confirmed, femoral artery ligation and excision was performed to induce ischemic skeletal muscle injury. Interestingly, 10 days after ischemic insult, assessment of all regenerated myofibers, defined by their centralized nuclei, revealed the presence of GFP+ myofibers. These...
Myh11⁺-derived GFP⁺ regenerated myofibers, found amongst neighbouring RFP⁺ regenerated myofibers, were seen consistently in wild-type C57BL/6J mice after ischemic injury. Notably, Myh11⁺-derived myofibers were structurally similar to RFP⁺ satellite cell-derived myofibers in cross sectional areas and sarcomere lengths. This adds to the growing body of evidence that have shown that a population of pericytes with multipotent potentials have myogenic capacities upon injury (Crisan et al., 2008; Dellavalle et al., 2011). To further assess for Myh11⁺ mural cell-derived regenerated myofibers, we undertook live animal imaging investigations via intravital fluorescence microscopy. We discovered endogenous in vivo expression of either TdTomato or eGFP signals throughout the full length of regenerated myofibers without the use of any exogenous fluorescent identification tools. This myofiber homogeneity is consistent with previously reported bidirectional proliferation, migration and fusion of activated myogenic progenitor cells in regenerating muscles (Webster et al., 2016). In their report, Webster et al. did not find any evidence of myogenic progenitor activation and expansion in the absence of myofiber injury. Similarly, my results show that eGFP signal was only found in regenerated myofibers and not in uninjured muscle tissues. Altogether, these results suggest the presence of Myh11⁺-derived pool of myogenic progenitor cells that are endogenous within muscle tissues that are activated upon ischemic injury.

I further explored possible endogenous regulators of mural cell reprogramming by studying the consequences of Sirt6 knockout in Myh11⁺ mural cells. By unbiased immunohistochemical methods, I discovered that Sirt6 knockout in Myh11⁺ mural cells severely impair this reprogrammed myogenic potential in the ischemic hindlimb. This is the first study to examine the effects of Sirt6 knockout in mural cells in relation to cellular reprogramming in the injured skeletal muscle. Prior studies have examined the effects of SIRT6 on reprogramming in dermal fibroblasts (Sharma et al., 2013) and adipocytes (Chen et al., 2017) where it is thought to be important for both gain of multipotency as well as terminal differentiation. Specifically, Sharma et al. showed that SIRT6 in human dermal fibroblasts declines with age, which reduces reprogramming efficiency by Yamanaka factors. They then showed that this decline in gain of multipotency can be rescued by delivery of SIRT6 exogenously (Sharma et al., 2013). Similarly, Chen et al. showed that Sirt6 in preadipocytes regulate mitotic clonal expansion, which is essential for terminal
differentiation into mature adipocytes (Chen et al., 2017). Future studies to examine the
effects of SIRT6 activating compounds or even exogenous delivery of SIRT6 to pericytes
may provide valuable clues toward therapeutically exploiting their myogenic potential.

There are limitations to the generalizability of our data. This is the first report that I am
aware of that examines the reprogrammed myogenic potential of vascular mural cells in an
ischemic model of muscle injury. Although previous studies have investigated this
phenomenon in other injury models such as barium chloride induced muscle injury
(Guimaraes-Camboa et al., 2017) and cardiotoxin induced injury (Dellavalle et al., 2011),
these direct injection models are susceptible to toxin-induced side effects on myogenic
progenitor cells and the vasculature. Indeed a comparison of the various models of skeletal
muscle injury and regeneration showed that both chemical injury by barium chloride as
well as cardiotoxin-induced injury caused significant loss of satellite cells (Hardy et al.,
2016). Therefore, ischemia models that avoid toxin-induced cellular injury may be more
clinically relevant to PAD.

Due to the genetic constraints of the Y-chromosome-embedded Myh11-Cre allele (Herring
et al., 2014; Wirtth et al., 2008), we were only able to perform experiments on male mice.
It will be important to investigate mural cells in female mice to test for sex differences
(Peng et al., 2011). Furthermore, our hindlimb ischemia studies were limited to young and
otherwise healthy mice without the presence of comorbidities. This is a common limitation
of the mouse hindlimb ischemia model (Nowak-Sliwinska et al., 2018), which can be
somewhat mitigated with future studies that use older mice fed disease-inducing diets.
Similarly, our mice studies were limited to the C57BL/6 genetic background. Other mouse
strains may produce differing degrees of injury and regeneration, which may affect the
myogenic potential of native pericytes (Helisch et al., 2006; S. M. Marques et al., 2011).

In our reporter mouse system, the Myh11 reporter could theoretically mark: i) pericytes
that have differentiated, ii) pericytes that may enter the stem cell niche , iii) SMCs, iv)
another cell population that could transiently turn on the Myh11 promoter elements in the
transgene. However, GFP expression was not seen in any non-injured or non-vascular
tissues .
In summary, we have identified a program of Myh11⁺ cell-derived skeletal myogenesis following ischemic injury. This is an infrequent but autonomous process, establishing complexity in the stem cell response to ischemic injury. Determining whether this Myh11⁺ progenitor cell-based program contributes uniquely to muscle regeneration, and whether it can be therapeutically amplified, will be important for efforts to reduce the burden of PAD.

3.5 References


resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. *Nat Commun*, 2, 499. doi:10.1038/ncomms1508


the design and understanding of ischemia models. *PLoS One, 8*(12), e84047. doi:10.1371/journal.pone.0084047


Gender differences affect blood flow recovery in a mouse model of hindlimb ischemia. *Am J Physiol Heart Circ Physiol, 300*(6), H2027-2034. doi:10.1152/ajpheart.00004.2011


CHAPTER 4

4 Myh11^{+} Specific Mural Cell Knockout of Sirt6 Leads to Senescence that Impairs Regeneration of the Ischemic Mouse Hindlimb and the Associated Microvascular Network

4.1 Introduction

Cardiovascular diseases are the leading cause of mortality world-wide (Lozano et al., 2012). Among them, peripheral artery disease (PAD) has the second fastest growing mortality rate globally, behind only atrial fibrillation/flutter (Lozano et al., 2012; Song et al., 2019). Between 1990 and 2010, PAD mortality rates increased by 167%; during the same time period, ischemic heart disease grew by 35% and cerebrovascular disease by 26% (Lozano et al., 2012; Song et al., 2019). Interestingly, PAD is biologically distinct from cardiac and cerebral vascular diseases due to the ability of skeletal muscles to regenerate upon injury. This regenerative capacity raises prospects for therapeutically reversing tissue damage and restoring tissue structure and function.

Because microvessels can regenerate after ischemic muscle injury, therapeutic angiogenesis for PAD has been extensively explored (Annex, 2013; Iyer et al., 2017). This therapeutic paradigm has yielded promising results in the pre-clinical setting (Annex, 2013; Vale et al., 2001). Unfortunately, the pre-clinical successes have not translated well to clinical results for patients suffering from PAD. This translational gap may be attributable to several factors, including suboptimal delivery strategies and intrinsic biases with animal models (Lee et al., 2020). In addition, recent evidence suggests that the microvascular network that regenerates following injury may not be optimized in structure or function, with defects in network hierarchy and mural cell investment (Arpino et al., 2017). A better understanding of the molecular controls over mural cells during microvascular regeneration in ischemic muscle is required.

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases that have major regulatory roles in multiple pathways, in response to nutrient sensing (Haigis et al., 2010). Defects in NAD processing have been shown to significantly
disrupt vascular smooth muscle cell homeostasis and vascular integrity (Watson et al., 2017) while enhancement of intracellular NAD and sirtuin 1 activity improved capillary density and blood flow recovery following hindlimb ischemia (Wang et al., 2014). Within the seven member family of sirtuin enzymes, sirtuin 6 (Sirt6) has been recently discovered and there are lethal consequences to its absence (Mostoslavsky et al., 2006). In addition to its function as a histone deacetylase, Sirt6 has also been shown to function as an ADP-ribosyltransferase and de-fatty-acylase (Chang et al., 2020). The multi-functional capabilities of Sirt6 underlies its regulatory roles in several important cellular processes like glycolysis, gluconeogenesis, inflammation and tumorigenesis (Chang et al., 2020; Tasselli et al., 2017), depending on cell type and context. In large vessels like the aorta, vascular smooth muscle cell-specific ablation of Sirt6 has been shown to induce senescence and exacerbate atherosclerosis (Grootaert et al., 2021). However, the role of Sirt6 in the regenerating microvasculature after hindlimb ischemia has not yet been studied.

The use of transgenic reporter mice is a powerful means of investigating specific cell populations \textit{in vivo}. Transgenic mice defined by the smooth muscle myosin heavy chain, Myh11, promoter have been found to reliably label vascular smooth muscle cells and pericytes (Hess et al., 2019). This allows for targeted exploration of the molecular pathways that may be vital to effective vascular regeneration. Therefore, we sought to investigate the effects of Sirt6 knockout in vascular mural cells using Myh11-CreERT$^2$ reporter mice. First, we bred mice harboring a Myh11-CreERT2 allele into R26-mT/mG mice, which was then crossed with mice carrying a floxed Sirt6 gene, and, finally backcrossed to a C57BL/6 background for at least 10 generations. These mice underwent femoral artery ligation and excision for analyses of muscle regeneration and angiogenesis by histological and intravital microscopy methods. These findings uncover a novel role for Sirt6 in perivascular mural cells and provide new potential targets for skeletal muscle regeneration following ischemic injury.
4.2 Materials and Methods

4.2.1 Generation of a Vascular Mural Cell-Specific Sirt6-Deficient Fluorescent Reporter Mouse

Experiments were conducted in accordance with the University of Western Ontario Animal Care and Use Subcommittee, which follows the policies set out by the Canadian Council on Animal Care. This study was also conducted in accordance with the Animal Research: Reporting In Vivo Experiments guidelines for transparent reporting of animal research (Kilkenny et al., 2010) and the 2018 consensus guidelines for the use and interpretation of angiogenesis assays (Nowak-Sliwinska et al., 2018).

We bred mice harboring a Myh11-CreERT2 allele (Wirth et al., 2008) into R26-mT/mG mice (Muzumdar et al., 2007) (Jackson stock #007676), both of C57BL/6 genetic background. The mice constitutively express a membrane-targeted TdTomato protein from the Rosa26 locus, but when Cre is activated the red fluorescence is replaced by that of membrane-targeted eGFP. Male mice carrying the Myh11-CreERT2 allele as well as R26-mT/mG alleles were then cross bred with Sirt6flox/flox females of FVB background (The Jackson Laboratory, Sirt6tm1.1Cxd/J, 017334) to produce male offspring that were heterozygous for floxed Sirt6 (Sirt6flox/wt) with Cre and mT/mG expression. These offspring were back-crossed with C57BL/6 mice for over 10 generations to produce Myh11-CreERT2, R26-mT/mG, Sirt6flox/wt mice of C57BL/6 genetic background. Finally, heterozygous offspring were mated together to generate homozygous floxed mice as well as littermate WT controls. To identify Sirt6flox/flox mice, Sirt6WT and floxed alleles are amplified using primers P1: 5’ AGT GAG GGG CTA ATG GGA AC 3’ and P2: 5’ AAC CCA CCT CTC TCC CCT AA 3’.

Eight-week-old mice received tamoxifen for 5 days. Four weeks after the last dose of tamoxifen the mice were subjected to right femoral artery ligation and excision, and muscles were evaluated 10 days or 28 days later. Mice were housed in clear, plastic cages containing standard bedding and maintained on a 12-hour light and dark cycle at 23°C and 50% relative humidity. Mice were fed water and normal mouse chow diet (Diet 2018; Harlan Teklad, Madison, WI), which were available ad libitum.
4.2.2  Mouse Model of Hind Limb Ischemia

Hind limb ischemia was induced by ligating the right femoral artery above and below the proximal caudal femoral artery (Kochi et al., 2013), also known as profunda femoris branch, using 6-0 silk sutures and excising the intervening 2- to 3-mm portion of artery (Arpino et al., 2017; Frontini et al., 2011; Lee et al., 2020; Limbourg et al., 2009).

4.2.3  Histologic Analysis of the Distal Hindlimb

Whole hind limbs were dissected, immersed in 4% paraformaldehyde overnight, and then decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days (Arpino et al., 2017; Lee et al., 2020). Each hind limb was embedded in paraffin and sectioned at the distal hind limb (the widest muscle portion of the below-knee tissue region). Skeletal muscle injury areas were identified for all muscles and regenerated myofibers were further distinguished by their centralized nuclei (Folker et al., 2013; Hall-Craggs, 1974; Harris et al., 1982).

Five-µm-thick full cross sections of hind limbs were stained for hematoxylin and eosin. Entire cross sections were digitally scanned using a Leica Aperio AT2 Digital Pathology Slide Scanner (Leica Biosystems, Germany) with 40× objective engaged. Skeletal muscle injury and non-injury areas were identified for all muscles, categorized as either normal, regenerated, or necrotic based on peripheral myofiber nuclei, regenerated myofibers with centralized nuclei, or disrupted myofibers devoid of nuclei respectively (Folker et al., 2013; Hall-Craggs, 1974; Harris et al., 1982). Total muscle infarct areas were calculated as the sums of regenerated and necrotic areas. Areas were quantified using ImageJ (National Institutes of Health) and Aperio ImageScope (version 12.3.2.8013; Leica Biosystems).

4.2.4  Assessment of Fibrillar Collagen Content in the Saphenous Artery by Circular Polarization Microscopy

The saphenous arteries within distal mouse hindlimb sections, located lateral to the popliteus muscle at the hindlimb surface, were assessed after staining sections with picrosirius red using a commercially available kit from Polysciences, Inc (Warrington, PA). Briefly, deparaffinized sections were incubated with 0.1% Sirius red F3BA in saturated picric acid for 30 minutes and rinsing twice with 0.01 mol/L HCl. Collagen
organization was assessed using circular polarization microscopy. Picrosirius red–stained sections of aorta were visualized and measurements were obtained using an Olympus BX51 microscope (Olympus Canada, Inc., Richmond Hill, ON, Canada) with polarizer-interference filters, a liquid crystal compensator, a charge-coupled device video camera, and Abrio software (Abrio LC-PolScope; Cambridge Research and Instrumentation, Inc., Hopkinton, MA). Mean collagen content was assessed in the medial layers of 10 uninjured mice and 13 injured-regenerated mice. The birefringent signal was corrected prior to final measurements based on total elimination of signal detected at the endothelial layer.

4.2.5 Laser Speckle Contrast Imaging Flow Analysis

Bulk blood flow to the distal hind limb was assessed using laser speckle contrast imaging (moorFLPI-2; Moore Instruments, United Kingdom). Relative limb perfusion was measured pre- and post-ischemia using regions of interest confined to the plantar soles, as reported previously (Limbourg et al., 2009). Mice were lightly anesthetized using isofluorane (1.3%–1.5% mixed with 100% oxygen at a rate of 0.8 L/min) and placed on a heating pad, covered by a black mat provided by the manufacturer, to maintain a body temperature of 36.5±0.5°C. The mice were acclimatized to the anesthetic and heating pad for 5 to 7 minutes before imaging and flow data collected according to manufacturer instructions. Image settings were set to Temporal Measure Mode with a Temporal Filter of 250 frames. Postprocessing of images and data extraction were performed using moorFLPI Review V5.0 software (Moore Instruments). Results are expressed as the ratio of perfusion in the ischemic (right) limb versus the nonischemic (left) limb.

4.2.6 Immunostaining and Angiogenesis Analysis

Whole hind limbs were dissected, immersed in 4% paraformaldehyde overnight, and then decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days (Arpino et al., 2017; Lee et al., 2020). Each hind limb was embedded in paraffin and sectioned at the distal hind limb (the widest muscle portion of the below-knee tissue region). Skeletal muscle injury areas were identified for all muscles and regenerated myofibers were further distinguished by their centralized nuclei (Folker et al., 2013; Hall-Craggs, 1974; Harris et al., 1982; Lee et al., 2020).
The skeletal muscle vasculature was evaluated by immunostaining for CD (cluster of differentiation) 31–positive endothelial cells. Five-micrometer sections were subjected to antigen retrieval (sodium citrate 0.01 M, pH 6.0 [Sigma], and heating with pressure in a 2100 Retriever device [Prestige Medical]) and immunostained using rat monoclonal anti-mouse CD31 antibody (1:20, Clone SZ31; Dianova). Bound primary antibody was detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:100; Thermo Fisher) and Alexa Fluor-594 conjugated goat anti-rabbit IgG (1:100; Thermo Fisher). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech; 0100-20). Fluorescence imaging was undertaken by widefield microscopy (Olympus BX-51) using the Northern Eclipse image acquisition and analysis software (EMPIX Imaging, Inc). Capillary densities for all muscles were quantified in 5 equally spaced high-powered fields per muscle territory, using ImageJ (National Institutes of Health).

To comprehensively interrogate for Myh11⁺ cellular lineage, full transverse 5-µm sections of decalcified hindlimbs were double-immunolabeled for TdTomato (mT) and eGFP (mG) using rabbit polyclonal anti-RFP antibody (1:100; Rockland, 600-401-379-RTU) and goat polyclonal anti-GFP antibody (1:200; Abcam, Ab5450) respectively. Senescence-associated marker p16 was detected by using primary rabbit polyclonal anti-p16 antibodies (sc-28260 1:50; Santa Cruz Biotechnology, Dallas, TX). Bound primary antibodies were detected using Alexa Fluor-488 conjugated donkey anti-goat IgG (1:100; Thermo Fisher, A11055) and biotin conjugated donkey anti-rabbit IgG (1:100; Jackson Immuno-Research 711-065-152) along with streptavidin Dylight-549 (1:00; Vector Labs, SA5549). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech; 0100-20). Fluorescence imaging of histological preparations were undertaken by widefield microscopy (Olympus BX-51) using the Northern Eclipse image acquisition and analysis software (EMPIX Imaging, Inc). Structural quantification of myofiber characteristics were performed using ImageJ (National Institutes of Health).

4.2.7 Fluorescent Intravital Microscopy

Skeletal myofibers and their associated microvascular network within a 50-µm-deep zone across the entire mouse extensor digitorum longus (EDL) surface was assessed by live fluorescent intravital microscopy. Briefly, mice were anesthetized using isofluorane
(1.3%–1.5% mixed with 100% oxygen at a rate of 0.8 L/min) and placed on a heating pad to maintain a body temperature of 36.5±0.5°C. To expose the EDL muscle surface, 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. The EDL was covered with an 8×8-mm glass coverslip and positioned face-down on the stage of an inverted fluorescence microscope (Nikon A1R Confocal Laser Scanning System, Nikon, Japan). After a 20-minute stabilization period, fluorescent images of the EDL were acquired using the Galvano scanning mode with lasers at 488, 561, and 640 nm excitation wavelengths as appropriate through the NIS Elements software (Nikon, Japan). Intravascular space was identified by intra-penile injection of AngioSense 680EX fluorescent imaging agent (7×10⁴ MW; 100 μL; NEV10054EX, PerkinElmer).

4.2.8 Statistics

Descriptive data are presented as mean ± SD. Comparative data are presented as mean ± SEM. Normal distributions were confirmed using D’Agostino and Pearson omnibus normality testing. Comparisons were made by 2 tailed t tests or ANOVA with Bonferroni post hoc test. Multiple t test corrections were performed using the false discovery rate approach with Q=5% (Glickman et al., 2014). The 2-stage step-up method of Benjamini et al (Benjamini et al., 2006) was used. Associations between the presence of angiogenesis and histological muscle injury status were tested using χ² analysis (Campbell, 2007). Data were analyzed using Prism 8 (version 8.4.2.679; GraphPad Software), and P<0.05 was considered significant unless otherwise stated.

4.3 Results

4.3.1 Vascular Mural Cell Specific Sirt6 Deficiency Reduces the Regenerative Capacity and Increases Skeletal Muscle Necrosis in the Distal Hindlimb

To investigate the effects of mural cell-specific Sirt6 knockout in the ischemic hindlimb in mice, tamoxifen-inducible transgenic reporter mice on a C57BL/6 genetic background were subjected to unilateral femoral artery ligation and excision. Specifically, mice were generated harboring homozygous Sirt6<sup>flox/flox</sup> alleles along with a Y-chromosome
embedded Myh11-CreERT2 allele, which enabled vascular mural cell-specific targeting of inducible knockout. Littermates with Sirt6WT/WT alleles, again carrying the Myh11-CreERT2 allele, were used as controls. Intraperitoneal administrations of tamoxifen dissolved in corn oil for 5 days were performed. Following a washout period of 4 weeks, surgical disruption of downstream blood flow via femoral artery ligations and excisions was assessed by laser speckle contrast imaging. In all cases, bulk blood flow in the injured hindlimb decreased below 25% of the contralateral uninjured hindlimb. Full transverse cross sections of decalcified distal hindlimbs were studied at 10 and 28 days post femoral artery excision (Figure 4.1). In total, 136 distal hindlimb muscles from 17 mice were examined at day 10 and 80 distal hindlimb muscles from 10 mice were analyzed at day 28.

At the day 10 time point, 80 distal anterior hindlimb muscles from 10 knockout mice along with an additional 56 muscles from seven control mice were examined following H&E staining. Infarct zones were defined as regions that had a combination of necrotic as well as regenerating muscle zones, which were identified by their lack of nuclei or centralized nuclei respectively. Conversely, non-infarcted zones were identified by their peripheral myofiber nuclei. Compared to their wild type littermate controls, Myh11+-Sirt6 knockout mice demonstrated a similar mean total infarct area (4.9 mm² vs. 7.5 mm², p = 0.22) (Figure 4.2). However, within the infarct zones, SIRT6 mice had a significantly higher necrotic area compared to controls (mean areas of 18.9 mm² vs. 7.4 mm², p = 0.011). A trend of severe injury defined by the absolute increase in necrotic muscle
Figure 4.1 Whole distal hindlimb cross-sectional analysis of ischemia-induced skeletal muscle injury following femoral artery ligation and excision.

Complete cross-sections of mid-distal hindlimbs of control and Myh11⁺-Sirt6 knockout mice at day 10 post femoral artery ligation and excision. Decalcified hindlimb tissues were fixed in 4% paraformaldehyde and paraffin blocked for each analysis.
Figure 4.2 Injury analysis of total infarct areas and necrotic areas within ischemic mid-distal hindlimb muscles.

A. The total injury infarct area across the whole distal hindlimb in Myh11\(^+\)-Sirt6 knockout mice (\(n = 76\)) and littermate controls (\(n = 50\)) at day 10 following femoral artery ligation and excision. B. Percentage of infarct area that was occupied by necrotic muscle. All data points represent individual muscles.
Figure 4.3 Comparisons of necrotic areas in individual distal hindlimb muscles at day 10 following femoral artery ligation and excision.

Necrotic areas expressed as a percent of the total infarct area of individual muscles at day 10 following ischemic injury. In every distal hindlimb muscle, Myh11\textsuperscript{+}-Sirt6 knockout mice displayed a trend toward greater necrosis and reduced regeneration of skeletal myofibers compared to littermate wild type controls.
area in the Myh11\(^+\)-Sirt6 knockout mice was seen across all eight distal hindlimb muscles (Figure 4.3).

To better understand the kinetics of muscle recovery in Myh11\(^+\)-Sirt6 knockout mice, the regenerated zone areas were also evaluated at day 28. Fifty-six distal anterior hindlimbs of seven knockout mice and 24 muscles across three control mice were studied. The total infarct areas at day 28 were comparable to the total infarct areas at day 10 and not different between control and Myh11\(^+\)-Sirt6 knockout mice. Remarkably, both Myh11\(^+\)-Sirt6 knockout mice as well as control mice displayed minimal residual necrosis at 1% each (p = 0.38). Despite this similar muscle recovery by day 28, a subset of Myh11\(^+\)-Sirt6 knockout mice suffered from necrotic toes of their hind paws, as identified by their black discoloration. Of the seven Myh11\(^+\)-Sirt6 knockout mice, three suffered from necrotic toes compared to none in the control group of three mice (p = 0.48). Overall, these results reveal a novel phenotype characterized by delayed tissue regeneration following ischemic injury in mural cell Sirt6 knockout mice.

### 4.3.2 Vascular Mural Cell-Specific Sirt6 Deficiency Increases Peri-microvascular and Medial Fibrosis in the Skeletal Hindlimb Following Ischemic Injury

I next determined the extent of fibrosis after injury in the hindlimb. For this analysis the three most necrotic muscles were identified based on percent necrotic area, which were the tibialis posterior, the EDL, and the tibialis anterior muscles. In order to avoid any potential bias in analyses based on regions of interest, whole muscle analyses were performed by large area slide scanning using the Leica AT2 slide scanner at 40× magnification. The area fraction of fibrotic areas, as identified by blue staining on trichrome treated slides, were calculated on a total of 36 muscle sections in 12 mice using the Leica Image Scope software.

Mural cell-Sirt6 knockout mice displayed significantly greater fibrotic area compared to the control group (7.3% vs. 3.8%, p < 0.0001), which was consistent across all distal hindlimb muscles examined (posterior tibialis: 7.8% vs. 3.8%, p = 0.0051; EDL: 8.3% vs. 4.5%, p = 0.028; tibialis anterior: 5.7% vs. 3.2%, p = 0.0123) (Figure 4.4). Interestingly,
Figure 4.4 Fibrotic response to injury within distal hindlimb skeletal muscle tissues 10 days following femoral artery ligation and excision.

A. Area-fraction of fibrosis based on trichrome-stained distal hindlimb muscles. B. Representative images of trichrome-stained distal hindlimb muscle sections 10 days following ischemic injury (BX51 Olympus). Black arrowheads indicate fibrotic areas.
Figure 4.5 Fibrosis within the distal saphenous artery following femoral artery ligation and excision and establishment of collateral flow.

A. Representative light microscopy (top) and circular polarized microscopy (bottom) images following picrosirius red staining of distal hindlimb muscles, 10 days after ischemic injury. White arrowheads indicate medial collagen. B. Fibrillar collagen content at baseline prior to injury followed by the injured state 10 days following femoral artery ligation and excision.
qualitative analyses revealed greater fibrotic accumulation around peri-microvascular areas. Therefore, further analyses of fibrosis within the vessel walls were studied. This required greater resolution and specificity of collagen content identification, which was achieved via picrosirius red staining.

The whole hindlimb analysis approach of distal cross-sections allowed for detailed examination of larger arteries, such as the saphenous artery, where collagen scarring can be measured in the smooth muscle cell-rich media. The saphenous artery in the murine model receives blood flow from remodeled collateral arteries following femoral artery ligation and excision (Meisner et al., 2013; Ramo et al., 2016). Here, we undertook picrosirius red staining to identify collagen deposition within the artery media. Imaging via polarized light microscopy was performed to identify collagen content. At baseline in uninjured limbs, there were no differences between Myh11\(^+\)-Sirt6 knockout mice and control mice (1% vs. 1%, \(p = 0.97\)). However, 10 days following femoral artery ligation and excision, the saphenous arteries in Myh11\(^+\)-Sirt6 knockout mice contained greater medial fibrosis compared to wild type controls (2.7% vs. 1.7%, \(p = 0.028\)) (Figure 4.5). Therefore, Myh11\(^+\)-Sirt6 knockout in mural cells was shown to produce a fibrotic phenotype during post-ischemia remodeling of skeletal muscle as well as arterial walls.

### 4.3.3 Blunted Bulk Flow Recovery in the Myh11\(^+\)-Sirt6\(^{null}\) Mouse Hindlimb

I next analyzed vascular flow recovery in mice with vascular mural cell-specific deletion of Sirt6. Laser speckle Doppler imaging and bulk flow analyses were performed, comparing the ischemic limb to the non-ischemic limb to obtain relative blood flow measurements (Figure 4.6A). In total, 54 mice were studied over 10 days (\(n = 43\)) and 28 days (\(n = 11\)) following femoral artery excision.

Measuring relative bulk flow at the ventral hind paws revealed equal baseline blood flows between knockout mice and control mice. Immediately following femoral artery ligation and excision, all 54 mice were confirmed to have reduced bulk blood flow to the distal hindlimb, down to at least 25% of baseline measurements. Thereafter, we discovered that Myh11\(^+\)-Sirt6 knockout mice had blunted bulk blood flow.
Figure 4.6 Bulk blood flow analysis by speckle laser doppler flow imaging.
A. Representative images obtained by laser speckle doppler flow analyses following femoral artery ligation and excision. B. Time course assessment of the bulk blood flow recovery following successful surgical induction of ischemia by femoral artery excision.
recovery compared to injured wild-type littermate controls. This difference was statistically significant by day 10 (46.4% vs. 63.5%, \( p < 0.0001 \)). Blunted blood flow recovery to the distal hindlimb continued through day 14 (45.3% vs. 62.4%, \( p = 0.02 \)), day 21 (51.9% vs. 71.9%, \( p = 0.014 \)), and day 28 (51.0% vs. 71.8%, \( p = 0.012 \)) post-femoral artery excision (Figure 4.6B). Therefore, unlike the delayed regeneration observed within the skeletal muscles, vascular recovery appears to suffer from an altogether blunted recovery in Myh11\(^+\)-Sirt6 knockout mice compared to their littermate controls.

### 4.3.4 Altered Angiogenesis in Myh11\(^+\)-Sirt6\(^{null}\) Mice Subjected to Hindlimb Ischemia

I next examined the microvascular regenerative response of injured skeletal muscles in Myh11\(^+\)-Sirt6 knockout mice. For this analysis, 60 muscles from 20 mice were studied at day 10, along with an additional 30 muscles from 10 mice were examined at day 28. Five-\(\mu\)m muscle sections from 11 knockout mice and 9 control mice were immunostained for CD31 at day 10 and microvessel density was quantified. Seven knockout mice with 3 controls were similarly studied at day 28. Regions of interest were identified by centralized myofiber nuclei, indicative of regenerated muscle zones, in order to ensure neovessels were counted and not pre-existing vessels in uninjured territories.

The mean baseline capillary density in uninjured Myh11\(^+\)-Sirt6 knockout muscle sections was 839 capillaries / mm\(^2\) compared to 836 capillaries / mm\(^2\) in control mice (\( p = 0.977 \)). At day 10 following ischemic injury, Myh11\(^+\)-Sirt6 knockout mice were found to have a significantly lower capillary density compared to their controls at day 10 (1817 capillaries / mm\(^2\) vs. 2205 capillaries / mm\(^2\), \( p = 0.036 \)). By day 28 post-femoral artery excision, the capillary densities in both groups were once again equal (Myh11\(^+\)-Sirt6 KO: 1272 capillaries / mm\(^2\) vs. Control: 1134 capillaries / mm\(^2\), \( p = 0.436 \)). Thus, the angiogenic response of Myh11\(^+\)-Sirt6 knockout mice followed a similar pattern of altered regeneration as compared to the skeletal muscles. Despite the persistent blunted bulk blood flow recovery identified in Myh11\(^+\)-Sirt6 knockout mice, the angiogenesis response at 28 days after ischemic injury in Myh11\(^+\)-Sirt6\(^{null}\) mice was no different than that in injured control wild-type mice (Figure 4.7).
Figure 4.7 Baseline capillary density and angiogenesis response to ischemic injury within distal hindlimb skeletal muscles.

Capillary density before (top panel) and after (bottom panels) femoral artery ligation and excision. Myh11\(^+\)-Sirt6 knockout mice displayed reduced capillary density 10 days after ischemic injury. By 28 days after femoral artery excision, there was no significant difference in capillary densities between control and knockout mice.
Figure 4.8 Distal arterioles in Myh11\textsuperscript{+}-Sirt6\textsuperscript{null} mice display poor wrapping by vascular smooth muscle cells.

A. Representative images obtained via intravital fluorescence microscopy of arterioles wrapped by vascular smooth muscle cells in Myh11\textsuperscript{+}-Sirt6\textsuperscript{null} mice (right) and wild-type controls (left), both 10 days following femoral artery excision. B. Quantification of the fraction of arteriole length that is covered by smooth muscle cells. There is particularly poor coverage in Myh11\textsuperscript{+}-Sirt6 deficient mice. C. Unlike the regenerated arterioles, the regenerated capillary network showed similar pericyte wrapping between Myh11\textsuperscript{+}-Sirt6 knockout mice and their littermate wild type controls.
4.3.5 Abnormal Arteriole Wrapping in Myh11\textsuperscript{+}-\textit{Sirt6}\textsuperscript{null} Mice Following Hindlimb Ischemia

To further interrogate the basis of prolonged bulk blood flow dysfunction, live animal imaging via intravital fluorescence microscopy was performed. Previous studies have shown that the regenerated microvascular networks are characterized by abnormal mural cell wrapping, leading to flow dysregulation (Arpino et al., 2017). Therefore, by capitalizing on our innate mural cell-specific eGFP fluorescence, arteriole wrapping by vascular smooth muscle cells was examined. For this study, whole EDL muscles from 11 mice were surgically isolated and exposed for intravital fluorescence microscopy, as per previously established protocols. Using the Nikon A1R confocal microscopy system, 14 arteriole segments, of 8 µm – 20 µm in luminal diameter, from five Myh11\textsuperscript{+}-Sirt6 knockout mice and 24 arteriole segments from six corresponding littermate controls were identified for analyses. Smooth muscle cell coverage was determined by calculating the fraction of the length of the arteriole segments covered by eGFP positive smooth muscle cells relative to the entire linear length of the arteriole segment.

The mean mural cell coverage of regenerated arteriole segments within the wild type regenerated microvascular networks was measured to be 82.7%, similar to prior published reports (Arpino et al., 2017). This was visually evident by fluorescence gaps between eGFP fluorescing mural cells. In contrast, the mean mural cell coverage of arteriole segments in Myh11\textsuperscript{+}-Sirt6 knockout mice were reduced to 52.2% (p = 0.0002) (Figure 4.8). Therefore, \textit{Sirt6} knockout in mural cells led to a profound, insufficiently-wrapped state of distal arterioles in hindlimb skeletal muscles.

4.3.6 Reduced Junctional Pericytes and Capillary Pericyte Bridges in the Regenerated Microvascular Network in Myh11\textsuperscript{+}-\textit{Sirt6}\textsuperscript{null} Mice

I next sought to determine the extent of pericyte investment within the capillary network in the ischemic hindlimbs of Myh11\textsuperscript{+}-Sirt6 deficient mice. Live animal intravital fluorescence microscopy methods were again employed to accurately visualize the Myh11\textsuperscript{+} pericyte populations. Others have recently reported Myh11\textsuperscript{+} lineage tracing systems’
abilities to accurately identify more than 80% of the total hindlimb pericyte population in the mouse (Hess et al., 2019). For this study, 352 capillary regions of interests in five Myh11\(^+\)-Sirt6 knockout mice along with 382 capillary regions of interests in six control mice were carefully analyzed using 3D reconstructed images of regenerated hindlimbs.

At day 10 post-femoral artery excision, 87.7% of the regenerated capillary network in wild-type control mice was covered by pericytes and 89.8% in Myh11\(^+\)-Sirt6 knockout mice (p = 0.733). However, despite the equal overall pericyte content within the regenerated capillary network, we observed that there were differences specifically in those pericytes localized to capillary bifurcations – so called junctional pericytes (Gonzales et al., 2020). A junctional pericyte is defined as a pericyte whose nucleus is within 8-μm of the center of a capillary bifurcation. In control mice, 75.5% of capillary junctions contained at least one junctional pericyte. However, in Myh11\(^+\)-Sirt6 knockout mice, only 47.0% of capillary junctions contained at least one junctional pericyte (p = 0.0003). Another specialized, site-specific pericyte is referred to as a pericyte bridge, which physically links two adjacent capillaries. Pericyte bridges were reduced in Myh11\(^+\)-Sirt6\(^{null}\) mice by nearly 50% compared to wild type littermate controls. Within 266,161,500 µm\(^3\) of regenerated skeletal muscle volume in the control mice and 198,977,250 µm\(^3\) in Myh11\(^+\)-Sirt6 knockout mice, we identified 85 pericyte bridges per 100 µm\(^3\) compared to only 46 pericyte bridges per 100 µm\(^3\) of regenerated skeletal muscle (p = 0.0422) (Figure 4.9).

Interestingly, recent reports have suggested flow regulatory roles for junctional pericytes in other tissues such as the brain (Gonzales et al., 2020). Similarly, pericyte bridges have been implicated in the microvascular dysfunction of diseases like diabetes mellitus, which can be modulated by medications that normalize blood glucose concentrations (Corliss et al., 2020). The current findings suggest that the emergence of these site-specific pericytes in a regenerated microcirculation may be particularly dependent on Sirt6.
Figure 4.9 The regenerated capillary network in Myh11\textsuperscript{+}-Sirt6 knockout mice have reduced junctional pericytes and pericyte bridges, 10 days after ischemic injury.

A. Representative intravital fluorescence microscopy images of the regenerated capillary network, highlighted by eGFP\textsuperscript{+} mural cells. Arrow heads indicate pericyte bridges. Arrows indicate junctional pericytes. B. Quantification of junctional pericytes and pericyte bridges between Myh11\textsuperscript{+}-Sirt6 knockout mice and corresponding controls.
4.3.7 Myh11⁺-Sirt6 Deficient Mural Cells Upregulate p16 dependent Senescence Pathways

Mural cell-Sirt6 has been shown to regulate cellular senescence in various tissues, including the atherosclerotic large vessels (Grootaert et al., 2021). Therefore, we immunostained hindlimb regenerated muscle sections for p16^{Ink4a}, a marker for and mediator of cellular senescence in the vasculature (Balint et al., 2019; Watson et al., 2017). Tissues were co-stained for eGFP to label vascular mural cells, including pericytes. Overall, 70 mural cells across 5 control mice along with 75 mural cells across 5 Myh11⁺-Sirt6 knockout mice were studied using 5 µm thick cross-sections.

Remarkably, in Myh11⁺-Sirt6 knockout mice, p16^{Ink4a} was strikingly upregulated in eGFP⁺ mural cells compared to their wild type controls. Specifically, an average of 55% of Myh11⁺-Sirt6 knockout pericytes were identified to be p16 positive, compared to 1% in control pericytes (p = 0.0079) (Figure 4.10). In fact, four of five control mice did not have any detectable p16 signal within the nucleus of pericytes, while every Myh11⁺-Sirt6 knockout mice contained detectable p16 signal with the lowest population of p16 positive pericytes at 10% of total measured pericytes. Therefore, we have identified a senescence mediated correlation between Myh11⁺-Sirt6 knockout in vascular mural cells and abnormal hindlimb regeneration in ischemic skeletal muscle tissues.
Figure 4.10 Mural cell knockout of Sirt6 is associated with upregulation of p16\textsuperscript{Ink4a}, 10 days following femoral artery excision.

Top panels show representative images of control (left) and knockout (right) hindlimb sections co-immunostained for eGFP and p16\textsuperscript{Ink4a} (BX51, Olympus). Arrow heads indicate p16\textsuperscript{Ink4a}+ eGFP-labelled mural cells. Bottom graph shows the quantification analysis of the p16\textsuperscript{Ink4a} abundance in mural cells from Myh11\textsuperscript{+}-Sirt6\textsuperscript{null} mice and their littermate controls.

* Red blood cell autofluorescence within vessel lumen.
4.4 Discussion

The regeneration of skeletal muscle and the associated vascular network upon ischemic injury is a complex phenomenon that has yet to be fully characterized, but holds the keys to immense therapeutic potential. In this chapter, I used the mouse hindlimb ischemia model system to take advantage of its innate regenerative abilities (Lee et al., 2020). Recent evidence has shown that one vulnerability in the regenerated microvascular network is flaws in the mural cell investment in arterioles, leading to dysregulated blood flow throughout the regenerated network (Arpino et al., 2017). Therefore, I sought to investigate the role of the epigenetic regulator, Sirt6, in vascular mural cells in the setting of ischemic injury and regeneration. I undertook a comprehensive examination of the effects of Sirt6 knockout in mural cells in a mouse hindlimb ischemia model. By combining lineage tracing methodologies, unbiased histological and immunohistochemical analyses, and modern live tissue imaging techniques, I identified Sirt6 as a novel intrinsic regulator of microvascular regeneration in skeletal muscle.

To accurately and reliably investigate the cell autonomous regulation of vascular mural cells in skeletal muscle tissues, lineage tracing methodologies using transgenic mouse models were generated. Here, vascular mural cells were permanently labelled using an established mouse system that depends on Y-chromosome embedded Myh11-Cre expression (Herring et al., 2014; Wirth et al., 2008) to facilitate excision of target floxed sites. These mice were cross-bred with R26-mT/mG fluorescent reporter mice, which enabled visual lineage tracing of mural cells. After inducing severe ischemic injury via femoral artery ligation and excision, the total infarct area was measured across the entire distal hindlimb transverse section for an unbiased analysis. This revealed equivalent injury extents between mural cell-Sirt6 knockout mice and littermate wildtype controls, which served as a measure of internal control. Interestingly, however, within the infarct areas of the distal hindlimbs, the composition of regenerating versus necrotic tissues were significantly different. In fact, Myh11+/Sirt6 knockout mice suffered from greater muscle necrosis and reduced regenerated myofibers compared to controls. Furthermore, this reduced regenerative capacity was associated with worse fibrosis, particularly in the perivascular and interstitial regions. Remarkably, even within the media of the saphenous
artery walls a fibrotic response was noted. These histological observations established a severe injury phenotype in the hindlimbs of Myh11\(^+\)-Sirt6 knockout mice.

The fibro-necrotic phenotype observed in the hindlimbs of Myh11\(^+\)-Sirt6 knockout mice was associated with a corresponding reduction in bulk blood flow recovery at day 10 post femoral artery excision. At the arteriolar level, as identified via intravital microscopy, the reduced bulk blood flow recovery at day 10 was associated with patchy and inconsistent mural cell wrapping of distal arterioles between 7 µm and 20 µm in size. Downstream of arterioles, capillary densities within the distal hindlimbs at day 10, as a marker of angiogenesis, were significantly reduced in Myh11\(^+\)-Sirt6 knockout mice. Furthermore, detailed examination of the capillary network via live animal imaging using 3D intravital microscopy methods revealed a reduction in pericyte wrapping specifically at capillary bifurcations, also known as capillary junctions and junctional pericytes (Gonzales et al., 2020). In addition to the reduction in junctional pericytes in Myh11\(^+\)-Sirt6 knockout hindlimbs, pericyte bridges, which can be identified by the lack of plasma dye markers, were quantified. Recent studies have identified pericyte bridges as a maker of microvasculature health in diabetic mouse models of disease (Corliss et al., 2020). Here, the number of pericyte bridges observed within the regenerated capillary network was also significantly reduced. Therefore, we have identified multilevel microvascular structural abnormalities caused by Sirt6 knockout in mural cells that ultimately contribute to reduced blood flow recovery and worse tissue damage.

Studies of whole mouse knockout of Sirt6 as well as tissue and cell specific analyses of Sirt6 signaling pathways have identified cellular senescence as a major contributor to accelerated aging (Mostoslavsky et al., 2006) and atherosclerosis (Grootaert et al., 2021). Therefore, I undertook immunohistochemical studies to identify a senescence-associated marker of disease. In regenerated hindlimbs of Myh11\(^+\)-Sirt6 deficient mice, p16\(^{ink4a}\) was significantly upregulated in pericytes in which Sirt6 had been knocked out, as labelled by our lineage tracing methods. This implies an aged mural cell phenotype in my Myh11\(^+\)-Sirt6 knockout model.
Nuclear Sirt6 has been shown to regulate the TGFβ signaling pathways by post-translational modification of SMAD proteins. Specifically, Sirt6 is involved in deacetylation of SMAD2 and SMAD3, which leads to suppression of SMAD2 and SMAD3 mediated signaling (Maity et al., 2020; Zhang et al., 2021; Zhong et al., 2020). In the absence of Sirt6, SMAD3 acetylation may be increased, which could result in increased TGFβ signaling (Maity et al., 2020). It will be interesting to see if such mechanisms are involved in the Myh11<sup>+</sup>-Sirt6 knockout ischemic hindlimb mouse model. The upregulation of TGFβ-mediated pathways are additionally intriguing in our mouse model in the context of poor arteriole wrapping and delayed skeletal muscle regeneration. Balint et al., have shown that TGFβ withdrawal is crucial for smooth muscle cell collectivization and healthy homeostasis (Balint et al., 2015). During skeletal muscle regeneration, TGFβ signaling has been shown to inhibit myoblast proliferation (Delaney et al., 2017; Xu et al., 2018). Sirt6 deficiency in vascular mural cells could also result in tissue fibrosis via enhanced TGFβ signaling. Finally, TGFβ is known to be a driver of cell senescence (Yin et al., 2016).

While our transgenic lineage tracing system allowed for novel discoveries regarding the relationships between vascular mural cells and the regenerated microvascular network and skeletal muscle tissues, there are limitations to the generalizability of our data. Due to the genetic constraints of the Y-chromosome embedded Myh11-Cre system (Herring et al., 2014; Wirth et al., 2008), we were only able to perform experiments on male mice. It will be important to further investigate the effects of Sirt6 in mural cells in female subjects to validate our findings across sexes (Peng et al., 2011). Furthermore, our hindlimb ischemia studies were limited to young and otherwise healthy mice without the presence of comorbidities. This is a common limitation of the mouse hindlimb ischemia model (Nowak-Sliwinska et al., 2018), which can be somewhat mitigated with future studies that use older mice fed disease inducing diets and/or other interventions. Similarly, our mice studies were limited to the C57BL/6 genetic background. Other mouse strains may produce differing degrees of injury and regeneration (Helisch et al., 2006; Marques et al., 2011), which may affect the interplay between vascular mural cells and surrounding tissues. Finally, future studies aimed at examining the pericyte biology in relation to SIRT6 in human tissues are needed.
In summary, we have identified Sirt6 in vascular mural cells as a vital regulator of microvascular network and skeletal muscle tissue regeneration in the ischemic hindlimb. Senescence mediated pathways appear to be important in Sirt6 signaling, which may be amenable to pharmacologic intervention. Developing future therapeutic strategies at the intersection of nutrient sensing, epigenetic regulation, cellular reprogramming, and senescence may become more important in future translational studies.

4.5 References


the design and understanding of ischemia models. *PLoS One, 8*(12), e84047. doi:10.1371/journal.pone.0084047


CHAPTER 5

5 General Discussion and Summary

Understanding the cellular components and processes involved in tissue regeneration after damage is critical to advancing translational efforts toward the development of the next generation of targeted therapeutics for human diseases.

In this thesis, I have presented discoveries and concepts that advance our understanding of the relationships between regenerating skeletal muscles and the regenerating microvascular networks. First, I identified a previously unrecognized regional variability in regard to injury and angiogenesis that exists in the mouse hindlimb ischemia model of PAD. This led to the discovery of an exclusive relationship between angiogenesis and regenerating skeletal myofibers upon ischemic injury, and that the published literature to date may be exposed to this vulnerability. Second, I discovered that the mural cells of the microvasculature themselves can engage in skeletal myofiber regeneration through cellular reprogramming. Finally, I discovered a critical involvement of the histone deacetylase, Sirt6, in vascular mural cells in microvascular and skeletal myofiber regeneration. Specifically, I discovered that a loss of Sirt6 in vascular mural cells can lead to a severe necro-fibrotic phenotype that is associated with components of the senescence pathway and a blunted regenerative capacity. Collectively, these new findings uncover new details on the response to ischemic injury in skeletal muscle, a widespread phenomenon with high and increasing disease burden.

5.1 Regional Variabilities Within the Ischemic Mouse Hindlimb and Translational Vulnerabilities

The mouse hindlimb ischemia model of PAD has been widely used for understanding the cell and molecular biology of angiogenesis and skeletal muscle regeneration (Arpino et al., 2017; Couffinhal et al., 1998; Lee et al., 2020). This has been particularly true in the field of therapeutic angiogenesis where the hindlimb ischemia model has served as the predominant pre-clinical experimental system (Annex, 2013; Nowak-Sliwinska et al., 2018). However, translation from the mouse model of disease to clinical benefit for patients
has yielded poor results. This translational failure is likely multifactorial (Butler, 2008; Reis et al., 2008), which include previously unrecognized intrinsic vulnerabilities that may exist within the mouse model itself.

I discovered that, upon unilateral femoral artery ligation and excision, there exists a consistent regional variability in the pattern of skeletal muscle injury. More specifically, the anterior distal hindlimb muscles were consistently infarcted and regenerated, compared to the posterior distal hindlimb muscles and the upper hindlimb muscles, territories that only suffered partial injuries or no injury at all. Furthermore, regeneration of the hindlimb microvasculature via angiogenesis after an ischemic insult was exclusive to areas of active regeneration, and never found in necrotic areas or uninjured zones. Interestingly, a systematic analysis of the published literature to date revealed that the most commonly analyzed muscle was the gastrocnemius muscle, which is found in the posterior distal hindlimb where only a partial injury was observed. Remarkably, only 15% of all published reports examined angiogenesis in demonstrably regenerating muscle zones. Therefore, my findings reveal an important relationship between the regenerating microvasculature and corresponding skeletal muscle, while highlighting vulnerabilities and a proposed “disconnect” that exists in the current published literature. Attention to the mapping data that I acquired may improve translational efforts in future studies.

5.2 Future Directions in Translational Angiogenesis Studies

My findings on the relationship between regenerating microvessels and regenerating skeletal myofibers argue for quality assurance measures for future translational investigations. They also highlight the importance of elucidating mechanisms that govern the exclusive cellular connections between these two regenerating compartments. For example, the anatomical proximity between satellite cells and capillaries within skeletal muscles have been well characterized (Hendrickse et al., 2019). This site has been referred to as the vascular niche, a location where undifferentiated cells reside in quiescence (Latroche et al., 2015). However, in the setting of acute or chronic ischemia, the precise mechanisms of communication between satellite cells and neighboring capillaries are still unknown. Furthermore, the microvasculature is comprised of not only endothelial cells, but also perivascular mural cells. Therefore, the relationships between capillary endothelial
cells and perivascular pericytes with satellite cells and mature myofibers need to be further explored.

From an experimental perspective, our study has provided novel quality assurance measures that should improve reproducibility and perhaps translational efficacy. However, there are additional factors that may yet further improve the mouse hindlimb ischemia model and provide insights into personalized therapeutics in patients. For example, recent studies have highlighted the effects of maternal age and health in the future cardiovascular health of offspring (Cooke et al., 2019). It has been shown that advanced maternal age alone is a risk factor for poor cardiovascular health and reserve in the offspring in the adult stage of life. Such underrecognized risk factors, in addition to the traditional comorbid conditions like atherosclerosis and diabetes, need to be examined in both experimental systems and in translational studies for patients with PAD.

5.3 The Multipotent Potential of Vascular Mural Cells Upon Ischemic Injury

In addition to the exclusive tight linkage between angiogenesis and myogenesis in the ischemic hindlimb skeletal muscles of mice, I discovered a progenitor role for vascular mural cells upon ischemic damage. Specifically, using a fluorescent lineage tracing transgenic system, I found that vascular mural cells have the ability to give rise to newly formed skeletal myofibers after muscle infarct. This is a novel finding using mural cell identification via Myh11 expression, which has been shown to label both vascular smooth muscle cells and pericytes with high fidelity (Hess et al., 2019). These mural cell-derived regenerated myofibers within the ischemic hindlimbs were consistently found in all mice without any predilection for a specific muscle within the distal muscle bundle. Moreover, mural cell-derived myofibers appeared structurally identical to satellite derived regenerated myofibers in both sarcomere length and cross-sectional area. Live animal imaging via fluorescent intravital microscopy further revealed that mural cell-derived myofibers are of a homogeneous cellular lineage throughout the length of the muscle fiber, suggesting homologous fusion of Myh11+ -derived myoblasts. Therefore, these findings provide new evidence for pericyte-derived myogenesis in the adult hindlimb following ischemic injury.
5.4 Mural Cell Deletion of Sirt6 Alters Their Multipotent Capacity

Prior studies of Sirt6 in the whole mouse or other specific cellular compartments have identified other regulatory roles that include important functions in cellular reprogramming toward multipotent states (Etchegaray et al., 2015; Tasselli et al., 2017). My findings also revealed that in Myh11+/Sirt6null mice, compared to control wild-type mice, there was a significant reduction in pericyte derived regenerated myofibers following ischemic injury. In fact, in some knockout mice, there was a complete absence of mural cell-derived myofibers. Therefore, my findings overall reveal a new regulatory pathway by which regeneration of skeletal muscle tissues can be affected by Sirt6 deficiency in vascular mural cells.

5.5 Future Directions with Mural Cell Reprogramming and Multipotency Following Ischemic Injury

Although my findings add new evidence in support of mural cell reprogramming and multipotent capabilities, questions remain to fully capitalize on their potential. First, only a small subset of mural cells in each muscle were found to reprogram and participate in myogenesis. Identification and characterization of this subset is critical to understanding the molecular pathways that govern this process, and capitalizing on the findings for therapy. Furthermore, it is unknown if mural cells transdifferentiate directly into myoblasts and eventually myofibers, or if there is an intermediate state such as a mural cell-derived satellite cell or a mesenchymal stem cell that forms prior to myogenesis. The molecular pathways involved in mural cell reprogramming and fate determination needs to be further explored. Finally, whether there are pharmacologic, biologic, or other interventions that can amplify the pericyte-myocyte program will be important to ascertain.

5.6 Loss of Sirt6 in Vascular Mural Cells Cause Necro-Fibrosis and Reduced Tissue Regeneration

Productive tissue responses to damage, including regeneration after injury, depends on the cells’ abilities to adapt to environmental stress and maintain or acquire specialized functions. In the case of PAD in patients and the ischemic hindlimb in mice, cells within the affected limbs must adapt to the switch from a highly active metabolic state to an
ischemic environment with limited oxygen and nutrient supply. Innate regulators of cellular metabolism in response to nutrient availability include the sirtuin family of histone deacetylases (Haigis et al., 2010). In my study of Sirt6 in vascular mural cells, in the setting of hindlimb ischemic injury, I discovered that the loss of Sirt6 in vascular mural cells lead to a severe injury phenotype compared to wild type controls. More specifically, despite similar infarct areas, Myh11\(^+\)-Sirt6 knockout mice suffered greater necrosis of skeletal muscle tissues and reduced myofiber regeneration at 10 days post femoral artery excision. Furthermore, the reduced regenerative capacity of Myh11\(^+\)-Sirt6 knockout mice was associated with an increase in perivascular and interstitial fibrosis. Therefore, I have shown that perturbations in vascular mural cell Sirt6 activity results in detrimental consequences for skeletal muscle regeneration after ischemic injury. Conversely, Sirt6 is critical to effectively respond to severe ischemic injury.

5.7 Abnormal Microvascular Regeneration in Mice with Mural Cell Sirt6 Deficiencies

In order to further understand the association between Sirt6 knockout in mural cells and the resultant necro-fibrotic phenotype after injury, I examined the revascularization program, including microvascular regeneration, in the ischemic hindlimb. I discovered that Myh11\(^+\)-Sirt6 knockout mice suffered from diminished bulk blood flow recovery at day 10 post femoral artery excision compared to wild type littermate controls, as measured by laser speckle doppler flow imaging. Interestingly, this difference was evident by day 10 post ischemic insult and persisted to day 28.

At the microscopic level, analysis revealed significantly reduced capillary densities in Myh11\(^+\)-Sirt6 knockout mice compared to controls. This reduced angiogenic response corresponded with blunted skeletal muscle regeneration and a relative abundance of necrotic myofibers that had not yet been cleared. In addition, I found abnormal VSMC wrapping of pre-capillary arterioles, which implies poor VSMC collectivization, and altered pericyte investment in capillary networks. Specifically, I discovered that Myh11\(^+\)-Sirt6 knockout mice suffered from a significant reduction in junctional pericytes as well as capillary bridges, both of which are postulated to regulate capillary blood flow and capillary network structure (Corliss et al., 2020; Gonzales et al., 2020). Interestingly, I also
discovered a significant upregulation of p16<sup>ink4a</sup><sup>+</sup> mural cells following hindlimb ischemic injury, suggestive of cellular senescence, which may be contributing to the overall phenotype. Therefore, my findings reveal a critical role for Sirt6 in several aspects of mural cell organization with the potential for functional microvascular consequences, including dysregulation of blood flow control in response to metabolic demands.

5.8 Future Directions in the Regulatory Roles of Sirt6 in the Vascular Mural Cell

While the consequences of <i>Sirt6</i> knockout in mural cells during tissue ischemia have been characterized in the mouse hindlimb, more studies are needed to determine translational relevance. First, SIRT6 expression in human skeletal muscle, particularly in the vasculature, should be examined. One study using human dermal fibroblasts from young and older participants revealed that SIRT6 levels decline with age (Sharma et al., 2013). Furthermore, the effects of SIRT6 loss in these dermal fibroblasts were recovered by delivery of SIRT6 in the older age group. Thus, a careful investigation into SIRT6 delivery to cells may yield additional information regarding potential therapeutic benefits.

Second, my results revealed an upregulation of a senescence marker, p16<sup>ink4a</sup>, in the vascular mural cells of the regenerated microvasculature following ischemic injury. Thus far, SIRT6 has been shown to contribute to senescence in aortic smooth muscle cells during atherosclerosis (Grootaert et al., 2021). Interestingly, senescence in VSMCs have been linked to altered TGFβ signaling pathways (Balint et al., 2019; Yin et al., 2016). As one of the most ubiquitous signaling molecules in the human body, TGF-β is a unique multifunctional growth factor that is vital to healthy tissue homeostasis and response to tissue damage. Specifically, within the vascular system, TGF-β has been shown to regulate cell multipotency and differentiation as well as maintenance of normal mural cell organization upon differentiation (Balint et al., 2015; Delaney et al., 2017; Xu et al., 2018). For example, withdrawal of TGF-β in mature vascular smooth muscle cells of the aorta was discovered to be critical to the collectivization of vascular smooth muscle cells, which is necessary for healthy aortic wall maintenance (Balint et al., 2015). In the regenerating skeletal muscle, TGF-β has been shown to inhibit myoblast proliferation (Delaney et al., 2017) and down regulation of the TGF-β and phospho-Smad2/3 pathway appear to be necessary for
functional muscle regeneration. Similarly, TGF-β regulates stem cells such as embryonic stem cells and hematopoietic stem cells to maintain their pluripotent state, which eventually requires down-regulation of TGF-β signaling in order to terminally differentiate (Xu et al., 2018). Interestingly, sirtuin enzymes like Sirt6 has been shown to post-translationally regulate TGF-β signaling via deacetylation of Smad2 and Smad3 transcription factors, which suppress their activity (Maity et al., 2020; Zhang et al., 2021; Zhong et al., 2020). Furthermore, Sirt6 deacetylase activity at key histone sites have also been shown to regulate TGF-β signaling genes (Maity et al., 2020; Zhang et al., 2021; Zhong et al., 2020). Therefore, further exploration of the senescence-TGFβ pathways may provide vital clues toward discovery of modifiable factors in regenerative medicine.

5.9 Summary

The data presented within this thesis describe the vital link between skeletal muscle regeneration alongside microvascular regeneration during ischemic muscle injury. Angiogenesis occurs exclusively in zones of active skeletal muscle regeneration, which may be regionally variable. This provides a new framework for quality assurances and future pre-clinical studies of therapeutic angiogenesis. Remarkably, microvascular mural cells also possess the capabilities to actively participate in reprogramming and generate new myofibers themselves, albeit in low abundance. This cellular reprogramming capacity requires Sirt6 in mural cells. In addition, Sirt6 in mural cells appear to have important roles on the microvessels themselves, ensuring adequate investment by a suitable population of VSMCs and pericytes. Without this there is poor perfusion and a necro-fibrotic phenotype following ischemic muscle injury. Collectively, these findings provide further insights into the interactions between microvascular structures and surrounding muscle tissues, and more specifically the critical role of mural cells during skeletal muscle and microvascular regeneration after ischemic injury.

5.10 References


Appendix A: Confirmation of Copyright Clearance for Reproduction of Scientific Articles

Systematic Interrogation of Angiogenesis in the Ischemic Mouse Hind Limb

Author: Jason J. Lee, John-Michael Arpino, Hao Yin, et al
Publication: ATVB
Publisher: Wolters Kluwer Health, Inc.
Date: Aug 13, 2020

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BACK CLOSE WINDOW
Appendix B: Ethics Approval for Animal Use

Western

2018-161:3:
AUP Number: 2018-161
AUP Title: Smooth Muscle Cells and Vascular Disease
Yearly Renewal Date: 02/01/2022

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2018-161 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western’s Senate MAPPS 7.12, 7.10, and 7.15
      http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
      http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html

2) As per UCAC’s Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
      http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
   http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care
Appendix C: Gene Writing Conventions

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For reference, see:
http://www.jci.org/kiosk/publish/genestyle
Curriculum Vitae

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Education

Ph.D.
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Awards and Scholarships

Alfred Jay Award for Biological Systems Research (2021)
- Awarded to a Medical Biophysics graduate student with the best first-author research paper in basic biological systems research during their graduate studies. (Value: $2,000)

Canadian Institutes of Health Research (CIHR): Vanier Canada Graduate Scholarship (Vanier CGS)
- Sirtuin 6 and Inflammatory Vascular Diseases. Awarded May 2018
- 3 years (36 Months) Doctoral Level Scholarship; $50,000 per year (CAD), $150,000 (CAD) total award.

Canadian Institutes of Health Research Doctoral (CIHR-D) Scholarship: Sirtuin 6 and Inflammatory Vascular Diseases.
- Awarded May 2018
- 3 years (36 Months) Doctoral Level Scholarship; $35,000 per year (CAD), $105,000 (CAD) total award. Declined in favour of the Vanier Scholarship.

Cobban Student Award in Heart and Stroke Research (2017)
- Established by Audrey and Delmar Cobban and matched by Robarts Research Institute, this award recognizes doctoral research in the area of heart and stroke disease (Value: $1,500)
Best Poster Award: Western University Department of Medicine Research Day
  - Sirtuin 6 Knockdown Causes a Degeneration of the Elastic Media in a Mouse Model of Spontaneous Aortopathy, May 2017. (Value: $500)

Canadian Institutes of Health Research (CIHR): Frederick Banting and Charles Best Canada Graduate Scholarship (CGS-M) in health research.
  - Awarded April 2016
  - 12 months Masters Level Scholarship, (Value: $17,500)

**Peer Reviewed Publications**


P. Joy Dunmore-Buyze, Charmainne Cruje, Zengxuan Nong, **Jason J. Lee**, John A. Kiernan, J. Geoffrey Pickering, Maria Drangova. 3D Vessel-Wall Virtual


**Book Chapters**