The Role of Endothelial FoxO Proteins in Coordinating Skeletal Muscle Recovery Following Hind Limb Ischemia

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Abstract

Peripheral artery disease (PAD) is a common manifestation of atherosclerosis and is characterized by an accumulation of plaque within the arteries supplying blood to the lower extremities. Distal to the occlusion, the ischemic muscle is deprived of oxygen and nutrients, lowering viable tissue health. Physiological healing encompasses microvascular growth (i. e. angiogenesis and arteriolargenesis) and an inflammatory cascade that ensures blood-flow recovery and muscle regeneration within the ischemic limb. Patients with PAD, however, experience an attenuated angiogenic response and unresolved inflammation that often impairs muscle regeneration. Endothelial cells (ECs) play a central role in orchestrating the response to muscle injury as it can communicate with numerous cells within muscle tissue and influence myogenic, inflammatory and vascular processes (i. e. angiogenesis). However, this potential has not been established within the context of post-ischemic muscle regeneration. By altering the biological profile of the EC in ischemic muscle, we can more concretely identify its specific role in the recovery time-line. Therefore, my thesis project targets EC-specific Forkead Box O (FoxO) 1 and 3 proteins because of their known role in mediating vascular homeostasis, pro-inflammatory potential as well as plausible anti-myogenic influences on muscle. However, the singular role of EC-FoxO1 and the combined role of EC-FoxO1 and 3 in modulating these processes has not been established in a model of severe muscle ischemia. I hypothesized that the depletion of EC-FoxO proteins will enhance blood flow recovery, microvascular remodeling and skeletal muscle regeneration following severe hind limb ischemia. Additionally, the combined depletion of EC-FoxO1 and 3 will more profoundly improve these responses compared to the single depletion of EC-FoxO1. To test this hypothesis, male mice with an EC-specific inducible depletion of FoxO1 (EC-FoxO1 KD), FoxO1 and 3 (EC-FoxO1,3 KD) as well as EC-FoxO1,3-expressing (Control) mice were subjected

to severe hind limb ischemia. At 4- and 14-days of ischemia, blood perfusion, inflammation, microvascular remodeling and muscle recovery were measured and compared between each mouse cohort. We establish that EC-FoxO1,3 KD mice have enhanced post-ischemic (a) microvascular growth (more capillarity and arterioles); (b) blood flow recovery of the paw and leg; and (c) relative increase in myofiber cross-sectional areas, signifying an enhanced potential for myofiber growth. These positive responses were also correlated with more accumulation of fibrotic tissue. Interestingly, all these outcomes were greater in magnitude in EC-FoxO1,3 KD compared to EC-FoxO1 KD mice, suggesting the combined depletion of EC-FoxO1 and 3 has a more profound role in influencing ischemic muscle outcomes. Overall, these results demonstrate that EC-FoxO proteins have the potential to coordinate vascular, myogenic and tissue remodeling events that characterize post-ischemic muscle recovery. These novel functions of EC-FoxO proteins can eventually be harnessed as a potential therapeutic target with the goal of promoting a more favorable PAD prognosis.

List of Publications

1. Nwadozi, E., Rudnicki, M., **DeCiantis, M.**, Milkovich, S., Pulbere, A., Roudier, E., Birot, O., Gustafsson, T., Ellis, C.G., and Haas, T.L. (2020a). High fat diet pre-conditioning improves microvascular remodeling during regeneration of ischemic mouse skeletal muscle. Acta Physiol. Oxf. Engl. doi: 10.1111/apha.13449.

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List of Abbreviations

AKT – Protein Kinase B
Angpt2 – Angiopoietin-2
ATG12 – Autophagy Related 12
ATP – Adenosine Triphosphate
Bax – Bcl-2-associated X Protein
Bcl-2 – B-cell Lymphoma 2
Bim – Bcl-2-like Protein 11
BNIP3 – Bcl2 Interacting Protein 3
C:F – Capillary-to-fiber Ratio
CAT – Catalase
CD – Capillary Density
CD – Cluster of Differentiation
CECs – Cardiac Endothelial Cells
CLI – Critical Limb Ischemia
Col13α1 – Collagen Type XIII alpha 1 Chain
CTX – Cardiotoxin
Dll – Delta-like Protein
DNA – Deoxyribonucleic Acid
EC – Endothelial Cell
ECM – Extracellular Matrix
EDL – Extensor Digitorum Longus
ELK-3 – ETS like-1 Protein
eNOS – endothealial Nitric Oxide Synthase
FAP – Fibro Adipogenic Progenitors
FGF – Fibroblast Growth Factor
FoxO – Forkhead box o
HGF – Hepatocyte Growth Factor
HIF-1α – Hypoxia-inducible Factor Alpha
HUVEC – Humen Umbilical Vein Endothelial Cell
ICAM-1 – Intercellular Adhesion Molecule 1
IFNγ – Interferon Gamma
IGF-1 – Insulin-like Growth Factor 1
IL – Interleukin
JNK – c-Jun N-terminal kinase
MMPs – Matrix Metalloproteinases
MST1 – Macrophage Stimulating 1
mTOR – Mechanistic Target of Rapamycin
MuSCs – Muscle Satellite Cells
Myf5 – Myogenic Factor 5
Myf6 – Myogenic Factor 6
MyoD – Myoblast Determination Protein
NF-kβ – Nuclear Factor kappa beta
NO – Nitric Oxide
OCT – Optimal Cutting Temperature

P21 – Cyclin-dependent Kinase Inhibitor 21
P27 – Cyclin-dependent Kinase Inhibitor 27
PAD – Peripheral Artery Disease
pAKT – Phospho-Protein Kinase B
pENOS – Phospho-endothelial nitric oxide synthase
PDGF-BB – Platelet-derived Growth Factor BB
PDGF-β – Platelet-derived Growth Factor beta
PDGFRβ – Platelet-derived Growth Factor Receptor beta
Phd2 – Hypoxia-inducible factor prolyl hydroxylase 2
PI3K – Phosphoinositide 3-kinase
PLGF – Placenta-like Growth Factor
qPCR – Quantitative Real-time polymerase chain reaction
ROS – Reactive Oxygen Species
siRNA – Small Interfering Ribonucleicacid
SMA – Smooth Muscle Actin
SOD2 – Superpxide Dismutase 2
TGF-ß1 – Transforming Growth Factor beta 1
THBS1 – Thrombospondin-1
TIMP – Tissue Inhibitor of Metalloproteinaises 1
TNFα – Tumor Necrosis Factor alpha
VCAM-1 – Vascular Cell Adhesion Protein 1
VEGFA – Vascular Endothelial Growth Factor
VEGFR-2 – Vascular Endothelial Growth Factor 2
VSMC – Vascular Smooth Muscle Cell
Wnt – Wingless-related Integration Site
αSMA – alpha Smooth Muscle Actin

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Chapter 1: Literature Review

1.1 Structure and Function of Skeletal Muscle

Skeletal muscle is a highly adaptive tissue and comprises 40% of total body weight (Frontera and Ochala, 2015). A functional muscle allows individuals to stay active and is a strong predictor of overall health and wellbeing (Biewener, 2016; Grimmer et al., 2019). The architecture of the muscle is characterized by a rigid but highly organized scaffold of myogenic cells and extracellular matrix (ECM) that provides structural stability (Haun et al., 2019; Kjaer, 2004). The predominant cell-type within the muscle are multinucleated myofibers with contractile properties (Jones et al., 2004). These mechanical processes require chemical energy which is predominantly derived from the breakdown of glucose and fatty acids via glycolytic or oxidative metabolism and requires sufficient plasma-derived oxygen and nutrients (Jones et al., 2004; Frontera and Ochala, 2015). As such, skeletal muscle is a metabolically demanding tissue and can greatly impact whole-body metabolism.

1.2 The Microvascular Network

Every blood vessel in the circulatory system is composed of highly specialized endothelial cells (ECs) (Aird, 2008). Vascular hierarchy begins with large-diameter (>10 mm) arteries originating from the heart that deliver large volumes of blood to the peripheral limbs. At the skeletal muscle, smaller muscular arteries start to branch into arterioles (40-250 μm), which are composed of a layer of both ECs and contractile vascular smooth muscle cells (VSMCs). VSMCs have contractile properties that change vessel diameter and, in effect, control the distribution of blood flow throughout the muscle. (Tuma et al., 2008). Blood from the arterioles then flows into the capillaries (5-10 μm) which are composed of only a single layer of ECs. This thin layer serves as an interface

between the blood plasma and the cells of peripheral tissues. Within the skeletal muscle, capillaries are positioned longitudinally and parallel to individual myofibers, ensuring optimal delivery of oxygen and nutrients via trans-endothelial diffusion. (Tuma et al., 2008; Pittman, 2011). The capillaries then drain into venules. Venules collect metabolic waste from myofibers and transport these materials into the venous circulation. Additionally, venules are the major site for immune cell recruitment and transmigration of immune cells from plasma into peripheral tissues during inflammation (Pober and Sessa, 2015). Altogether, the microvascular network not only supplies blood and nutrients to peripheral limbs but can also clear metabolic waste and facilitate inflammatory processes that work to sustain muscle health and homeostasis.

1.2.1 Arteriolar Function and Remodeling

The contractile properties of VSMCs can be controlled by metabolites released from working skeletal muscle (e. g. potassium as a vasodilator) and/or mechanical forces of blood flow (Sheng and Zhu, 2018; Dodge et al., 1994). For example, blood flow causes ECs to experience shear stress which stimulates cell-surface mechanoreceptors and the subsequent activation of endothelial nitric oxide synthase (eNOS). eNOS produces nitric oxide (NO), which diffuses from the EC into adjacent VSMCs to stimulate their relaxation and vasodilation, resulting in an acute decrease in shear stress (Durand and Gutterman, 2013). Artery diameters can also change as a result of adaptations to chronic blood flow (e.g. tangential shear stress) (Ward Michael R. et al., 2000; Chiu and Chien, 2011). Chronic elevation in shear stress is decreased to homeostatic levels through outward remodelling (vessel lumen enlargement). This occurs when EC NO inhibits proliferation and induces apoptosis of VSMCs (Tronc et al., 1996; Cooke and Dzau, 1997). On the contrary, prolonged low levels of shear stress are combated by inward remodelling (vessel lumen

narrowing). This is a result of ECs producing transforming growth factor beta (TGF-ß) which concomitantly increases VSMC proliferation (Mondy et al., 1997).

The number of arterioles may also increase in response to chronic shear stress, as seen in exercise-induced adaptations. Studies report that pre-existing capillaries can transition into arterioles following chronic skeletal muscle stretch or electrical stimulation (Hansen-Smith et al., 2001; Hansen-Smith et al., 1998). This was observed when extensor digitorum longus (EDL) cross-sections contained enhanced co-localization of fluorescently immunolabeled smooth muscle actin positive (αSMA) capillary structures, which is a marker of VSMCs and indicative of neo-arterioles (Hansen-Smith et al., 2001; Hansen-Smith et al., 1998).

1.2.2 Angiogenesis

Angiogenesis is the growth of new capillaries from pre-existing ones. The expansion of a capillary network is vital for many physiological processes, including embryonic development and the growth or regeneration of organ systems (e. g. skeletal muscle) (Potente et al., 2011). An insufficient capillary network can worsen the outcomes of ischemic pathologies such as myocardial infarction, stroke and peripheral artery disease, all of which are characterized by impaired oxygen and nutrient delivery to a given tissue. In contrast, the excessive growth of the capillary network can permit continued expansion of a tumor and exacerbate cancer outcomes. Therefore, angiogenesis must be tightly controlled so that it can meet the functional demands of the tissue (Carmeliet, 2003).

There are two general forms of angiogenesis: intussusceptive and sprouting angiogenesis:

Intussusceptive angiogenesis is also known as splitting angiogenesis and is stimulated by high vessel wall shear stress. The vessel splits into bifurcations as interstitial cells invade the vessel

lumen. This results in a unique morphology where numerous intraluminal walls (pillar-like microstructures), rather than elongated sprouts, are developed (Burri and Tarek, 1990). This form of angiogenesis can be observed in many biological contexts such as embryonic tissue development (e.g. bone and muscle) as well as established capillary networks within glandular tissue, muscle and tumors (De Spiegelaere et al., 2012).

Sprouting angiogenesis is the more common type of angiogenesis in post-embryonic development. This is when endothelial cells sprout outward toward an angiogenic stimulus, forming a new vessel. Several events must occur during this process including enzymatic degradation of the capillary basement membrane (which removes a physical barrier for EC movement), EC proliferation and migration as well as tube formation and stabilization. It is also known that sprouting angiogenesis occurs in portions of tissue that have inadequate perfusion, such as hypoxic regions devoid of microvessels. Ultimately, hypoxic zones are more enriched with pro-angiogenic factors such as Vascular endothelial growth factor (VEGFA) that can stimulate the proliferation and migration of ECs towards these regions (Adair and Montani, 2010; Gerhardt, 2008; van Hinsbergh and Koolwijk, 2008).

Cellular mechanisms of vessel growth: Under basal conditions, the EC is quiescent, however, it can transition into an active state when exposed to various pro-angiogenic growth factors (Potente et al., 2011). Upon activation, the ECs that form a vessel sprout can assume two unique phenotypes. The most distal cell is known as the endothelial tip cell which leads/guides the sprout forward via filopodia-mediated cell migration (Adair and Montani, 2010; Potente et al., 2011). Following the tip cell is the endothelial stalk cell that functions to elongate the vessel through EC

proliferation. It is well established that both these cell-types transition between tip and stalk cell phenotypes which is mediated by Notch and VEGFA signaling (Jakobsson et al., 2010). In general, endothelial cells "compete" for the tip cell position, which drives the growth of the sprout (Potente et al., 2011; Blanco and Gerhardt, 2013).

Research has given insight into the role of EC metabolism in regulating angiogenesis. Quiescent ECs are known to suppress both glycolytic and oxidative metabolism because non-proliferating cells do not need extensive energy (Potente and Carmeliet, 2017). A corollary of this effect is that endothelial cells will not consume oxygen and nutrients (e. g. glucose and amino acids) that need to be delivered to distal tissues, maintaining the efficiency of substrate delivery. When ECs are activated (during times of angiogenesis) both tip and stalk cells increase glycolytic and/or oxidative metabolism to facilitate migration and proliferation, respectively (Potente and Carmeliet, 2017).

1.3 Skeletal Muscle Angiogenesis

The local tissue environment can influence endothelial cell biology and angiogenic potential. Within the skeletal muscle, metabolic demand of individual myofibers must be paralleled with the supply of oxygen and nutrient rich plasma. This is first achieved through enhanced blood flow and subsequently with increases in microvascular (capillary and arteriole) content. Studies show that more oxidative myofibers are associated with more capillaries compared to glycolytic fibers (Ingjer, 1979). Additionally, both animal and human models of chronic exercise (high oxygen demand) are known to stimulate skeletal muscle angiogenesis (Egginton, 2009; Hermansen and Wachtlova, 1971). This is characterized by enhanced capillary-to-fiber ratios (C:F) and capillary densities (CD) in the working muscle groups (Gute et al., 1996). With higher capillarity, this leads

to elevated skeletal muscle oxygen consumption and oxidative capacity (Hepple, 2000; Richardson et al., 1999). Angiogenesis must also occur during skeletal muscle regeneration, when myofibers are subject to damage as a result of injury (e. g. blunt trauma and ischemia).

1.3.1 Angiogenic Regulators in Skeletal Muscle

In general, angiogenesis is controlled by the balance of pro- and anti-angiogenic factors that can intrinsically or extrinsically shift EC biology to favour capillary expansion or stasis. (Haas and Nwadozi, 2015; Olfert and Birot, 2011). In response to exercise or muscle injury, myocytes, immune cells and tissue-resident fibroblasts can release cytokines or growth factors that can communicate with ECs via paracrine or juxtacrine mechanisms (Newman et al., 2011; Olfert et al., 2010; Sunderkötter et al., 1994).

Pro-angiogenic factors: The following discusses some pro-angiogenic factors that have been studied within the context of activity/exercise-induced skeletal muscle angiogenesis. A well-established pro-angiogenic factor is Vascular Endothelial Growth Factor A (VEGFA). In response to an exercise or hypoxic stimuli, myofibers enhance the production and release of VEGFA within the local environment (Gustafsson et al., 2002). Through a paracrine mechanism, VEGFA binds to VEGF receptors of the endothelium and stimulates EC migration, proliferation and overall capillary growth (Neufeld et al., 1999; Wu et al., 2000). An intrinsic pro-angiogenic factor is endothelial nitric oxide (NO) (Morbidelli et al., 2003; Cooke and Losordo, 2002). Exercise and skeletal muscle activity leads to enhanced blood flow and subsequent EC shear stress which stimulates EC production of NO via the enzyme endothelial nitric oxide synthase (eNOS) (Milkiewicz et al., 2005; Morbidelli et al., 2003). Our group has shown that EC-NO can enhance the production of skeletal muscle VEGFA and subsequent vascular growth (Uchida et al., 2015).

Additionally, pharmacological inhibition of eNOS activity can impair metabolic- and blood flow-induced angiogenesis of skeletal muscle (Baum et al., 2004). Capillary growth is also dependent on the ability of ECs to migrate through the rigid compartments of the skeletal muscle extracellular matrix (ECM). The ECM is a structural scaffold and is a physical barrier that prevents EC migration and capillary growth. Therefore, another subclass of pro-angiogenic factors are matrix metalloproteinases (MMPs), which are matrix degrading enzymes that facilitate EC migration (Haas et al., 2000; Sang, 1998). Within the context of skeletal muscle activity and exercise, Koskinen et al. reported that high-intensity eccentric muscle contractions can increase MMP-2 and MMP-9 expression (Koskinen et al., 2002). Our group also demonstrated that chronically stimulated rat skeletal muscle had a significant increase in MMP production which coincided with enhanced angiogenesis (Haas et al., 2000). This is due to MMPs facilitating tip cell migration during sprouting angiogenesis by degrading the capillary basement membrane (Haas et al., 2000; van Hinsbergh and Koolwijk, 2008).

***Anti-angiogenic factors*:** In general, anti-angiogenic factors impair capillary expansion by shifting EC biology towards stasis rather than growth. Well established extrinsic factors are tissue inhibitor of matrix metalloproteinases (TIMPs) and Thrombospondin-1 (THBS1). TIMPs are soluble secreted factors that inhibit the action of MMPs, which impedes ECM degradation and subsequent EC migration (Brew and Nagase, 2010; Handsley and Edwards, 2005). This can occur when skeletal muscle is no longer active and the disrupted ECM begins to regenerate and regain its integrity. THBS1 is another ECM protein that can directly interact with ECs to inhibit EC cell-cycle progression and overall capillary growth (Gao et al., 2016; Iruela-Arispe et al., 2004). Lastly,

Forkhead Box O (FoxO) transcription factors are intrinsic anti-angiogenic regulators that will be discussed further in section 1.4 (Haas and Nwadozi, 2015).

1.4 Forkhead Box O Transcription Factors

Humans and mice produce four FoxO isoforms: FoxO1, FoxO3, FoxO4 and FoxO6 (Eijkelenboom and Burgering, 2013). It is reported that there are redundancies in the functionality of these isoforms and depending on which cell they are derived, they can serve multiple purposes (Eijkelenboom and Burgering, 2013; Webb and Brunet, 2014). In ECs, FoxO1 and 3 are the most abundant FoxO isoforms and are crucial regulators of vascular homeostasis (Potente et al., 2005). At a molecular level, the transcriptional activity of FoxO1 and FoxO3 proteins is controlled by their ability to enter or exit the nucleus as well as their accessibility to target genes. Nuclear-cytoplasmic shuttling is modulated by phosphorylation and the direction of this transport depends on specific kinase signaling pathways. For example, the phosphoinositide 3-kinase (PI3K) signaling pathway (stimulated by insulin and glucocorticoids) leads to the phosphorylation of FoxO proteins and the subsequent inhibition of nuclear import (Daitoku et al., 2011; Vogt et al., 2005). However, the Jun N-terminal kinase (JNK) signaling pathway (stimulated by oxidative stress) phosphorylates FoxO proteins to facilitate nuclear entry (Daitoku et al., 2011; Vogt et al., 2005). Lastly, nuclear FoxO proteins may be acetylated or deacetylated, which can modulate their ability to form transcriptional complexes and bind to target genes. Reports show that deacetylation can enhance the transcriptional activity of FoxO proteins (Daitoku et al., 2011; Vogt et al., 2005). Once bound to DNA, FoxO proteins can upregulate multiple genes that promote cell-cycle arrest (e. g. p21 and p27), apoptosis (e.g. Bim and ATG12), oxidant stress resistance (e. g. SOD2 and CAT) and autophagy (e. g. ATG12 and BNIP3) (Eijkelenboom and Burgering, 2013; Zhang et al., 2011).

1.4.1 FoxO Proteins and Angiogenesis

The molecular structure of both FoxO1 and FoxO3 share significant similarities in sequence homology, as the forkhead, nuclear localization, nuclear export and transactivation domains are located in similar positions (Wang et al., 2014). Additionally, most phosphorylation sites of FoxO1 and FoxO3 are conserved. Other similarities are related to their ability to regulate vascular homeostasis. Potente et al. demonstrated that the silencing of FoxO1 and FoxO3 genes led to increases in endothelial cell migration and tube formation *in vitro* (Potente et al., 2005). Additionally, we have previously shown that depleting endothelial FoxO1, 3 and 4 enhanced the capacity for exercise-induced angiogenesis in skeletal muscle (Slopack et al., 2014). Altogether, both *in vitro* and *in vivo* studies exemplify how FoxO proteins are anti-angiogenic regulators.

Some differences between FoxO1 and FoxO3 have also been reported. Harvested microvascular ECs from adipose tissue and human umbilical vein endothelial cells (HUVECs) showed higher expression of *Foxo1* compared to *Foxo3* (Potente et al., 2005; Rudnicki et al., 2018). Additionally, embryonic mice homozygous for FoxO1 deletion ($FoxO1^{-/-}$), but not $FoxO3^{-/-}$ mice, died during embryogenesis as a result of defects in vascular development (Hosaka et al., 2004). This trend can also be compared to Paik et al.'s study where *in vivo* hemangioma development was assessed and compared in mice with an EC-specific inducible depletion of FoxO1, FoxO3 and a combination of FoxO1, 3 and 4. Interestingly, hemangioma development was greater in EC-FoxO1- compared to EC-FoxO3 -depleted mice (Paik et al., 2007). However, hemangioma severity was greatest in EC-FoxO1, 3 and 4-null mice (Paik et al., 2007). Altogether, these studies suggest that FoxO1 is the predominant FoxO isoform that controls embryonic and post-natal vascular development.

However, the combined function of all FoxO isoforms can have a more profound effect in regulating vessel growth and vascular-dependent tumor progression.

Similarities and differences in EC-FoxO1 and 3 target genes have also been established in HUVECs. Genes induced by FoxO1 and FoxO3 silencing (independently) included eNOS and ELK-3 (pro-angiogenic factors) as well as collagen type XIII alpha 1 (Col13α1) which is involved in matrix remodeling. Additionally, anti-angiogenic factor angiopoietin-2 (Angpt2) expression was downregulated in FoxO1-silenced ECs but was not altered in FoxO3-silenced ECs. Lastly, pro-inflammatory and pro-angiogenic cytokine IL-8 was upregulated in FoxO3-silenced but downregulated in FoxO1-silenced ECs (Potente et al., 2005). Altogether, this highlights that FoxO1 and FoxO3 can target similar and/or different genes.

Numerous studies have also exemplified how EC-FoxO1 and EC-FoxO3 can singularly modulate EC biology with respect to angiogenesis. However, these studies cannot conclude mutually exclusive differences, as no direct comparisons between EC-FoxO1 and 3 were conducted. These studies are discussed below:

Endothelial FoxO1: It was demonstrated that EC-FoxO1 can couple EC metabolism and vascular growth. This was described through a cell signaling mechanism in which FoxO1 impairs the function of c-MYC, a master regulator of EC glycolysis, mitochondrial biogenesis and cell-cycle progression (Dang, 2013). As such, FoxO1-mediated blunting of c-MYC resulted in impaired retinal vascular growth (Wilhelm et al., 2016). EC-FoxO1 can also control EC polarity and cell migration during hypoxia-mediated sprouting angiogenesis. It was shown that extracellular hypoxia stimulated endothelial production of mitochondrial-derived ROS which in turn activated

the mammalian sterile 20-like kinase 1 (MST1) protein. Activation of MST1 led to the phosphorylation of FoxO1 which enhanced the production of cell polarity- and migration-associated proteins (tubulin, centrosomes, caveolin and lamellipodia). This activated ROS-MST1-FoxO1 axis was associated with enhanced EC migration and directionality (non-random travel) in a wound closure assay (Kim et al., 2019).

Endothelial FoxO3: It is reported that FoxO3 alone can mediate hypoxic stress-induced apoptosis of ECs. For example, when cardiac endothelial cells (CECs) were subjected to hypoxia, FoxO3 increased the expression of apoptosis-associated genes Bim, Bax and Bcl-2. In turn, this resulted in the apoptosis of cultured CECs (Zhang et al., 2013). Altogether, FoxO3 alone can mediate anti-angiogenic functions of ECs within the context of cellular hypoxia.

Altogether, EC-FoxO1 and EC-FoxO3 play crucial roles in vascular homeostasis and have redundant or mutually exclusive functions. This raises the point that depleting one EC-FoxO isoform may not fully mitigate their anti-angiogenic functions. By depleting one isoform, the actions of the other isoform may persist within the vascular endothelium.

1.5 Cells of the Skeletal Muscle Microenvironment

The skeletal muscle microvasculature is in a dynamic relationship with numerous cell-types within the muscle tissue. This skeletal muscle microenvironment (or micro-niche) is composed of myocytes, vascular ECs, immune cells (e. g. tissue-resident macrophages and mast cells), muscle satellite cells (MuSCs) and other interstitial cells such as fibroblasts and fibroadipogenic progenitors (FAPs) (Figure 1.1) (Bentzinger et al., 2013; Yin et al., 2013). These cells are spatially arranged in a tissue scaffold that optimizes cellular communication which serves to maintain tissue

homeostasis. Despite myocytes being the predominant cell type within the micro-niche, vascular ECs comprise a large component of cellular content. This is apparent when visualizing microvascular structures of skeletal muscle using histological techniques. For example, lectin staining of skeletal muscle cross-sections can illustrate the pervasive nature of EC content within a muscle (Cebasek et al., 2004). At the micro-level, the endothelium is positioned between the various cells of the skeletal muscle micro-niche, and can influence the overall functioning of these cells.

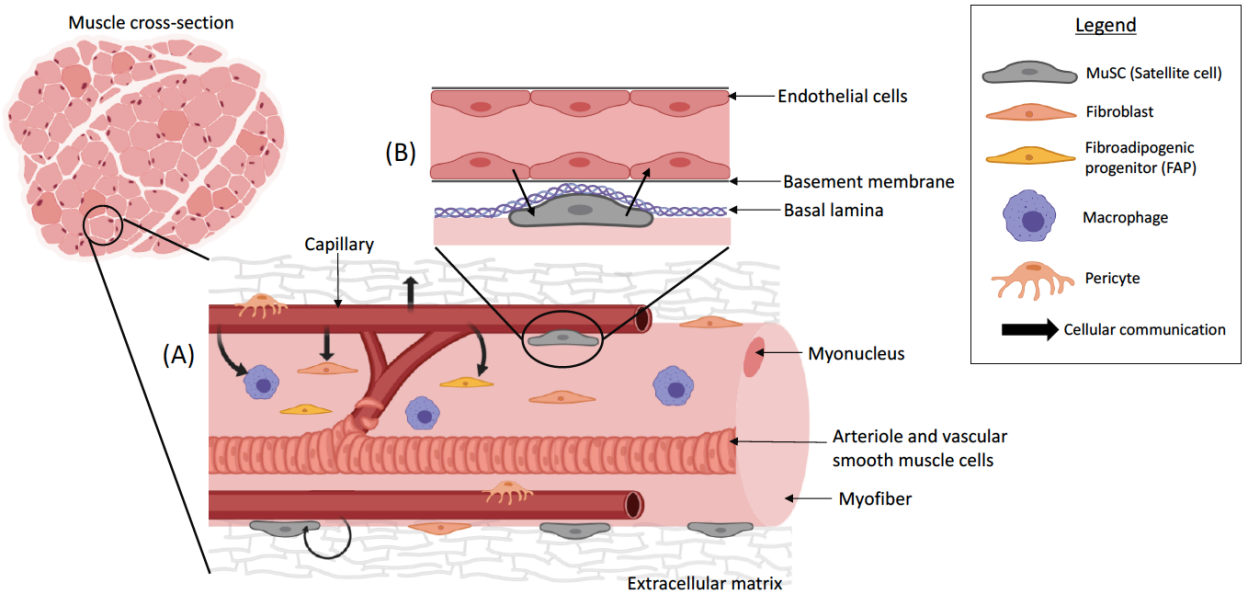

Figure 1.1: The Skeletal Muscle Micro-niche. (A) Schematic illustrating the communicative potential between ECs and numerous cells within the micro-environment. (B) Micro-anatomical position between vascular ECs and quiescent MuSCs, separated by the basal lamina and basement membrane. Created with BioRender.com.

1.5.1 Macrophages and Their Cytokine System

Adjacent to the ECs and located within the ECM are tissue-resident immune cells known as macrophages. Macrophages are phagocytes with a hematopoietic or tissue-specific origin and serve a general purpose of eliciting inflammatory processes that protect the tissue from foreign pathogens and injury (Kierdorf et al., 2015). Macrophages may also be derived from the plasma

in the form of monocytes that bind to venular ECs, transmigrate and home into a chemokine- and cytokine-rich tissue and subsequently differentiate into macrophages (Figure 1.2) (Jakubzick et al., 2017). In studies that deplete macrophage numbers after tissue trauma, inflammation and tissue regeneration is severely impaired, highlighting the indispensable functions of macrophages in muscle recovery (Brigitte et al., 2010).

It is well established that macrophages are a highly heterogeneous population and their phenotypes can vary depending on the tissue and cellular environment in which they reside (Gordon and Plüddemann, 2017). Collectively, macrophages secrete a variety of factors with distinct phenotypic characteristics and can alter the micro-cellular environment of the skeletal muscle. Macrophage phenotypes have generally been categorized as pro-inflammatory (M1) or anti-inflammatory (M2) (Tidball, 2017; Jakubzick et al., 2017). M1 macrophages promote the degradation and phagocytosis of cells that have been damaged as a result of tissue trauma (Wynn and Vannella, 2016). This is facilitated by the secretion of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1α and tissue necrosis factor alpha (TNFα) (Wynn and Vannella, 2016). These cytokines can recruit more pro-inflammatory immune cells to the region of tissue damage and/or directly influence the inflammatory profile of ECs. For example, studies show that TNFα, derived from M1 macrophages can stimulate the expression of EC leukocyte adhesion proteins such as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) (Pietersma et al., 1997). Additionally, M1 macrophages are a major source of reactive oxygen species (ROS), which can further induce tissue stress by promoting cell damage and feedforward inflammatory cascades (Tan et al., 2016). On the contrary, M2 macrophages are responsible for inflammatory resolution, phagocytosis and clearance of cellular debris and tissue regeneration.

This is achieved by secreting anti-inflammatory cytokines such as IL-10 and pro-regenerative growth factors such as TGF-ß and VEGFA (Wynn and Vannella, 2016). These proteins can mediate the phenotypic transition of M1 to M2 macrophages and stimulate adjacent ECs, satellite cells, myocytes and fibroblasts, which promotes vascular growth, myocyte maturation and tissue remodeling. By influencing myogenic and vascular cell populations, macrophages can play a major role in how the skeletal muscle responds to injury. A summary of these functions is depicted in Figure 1.2.

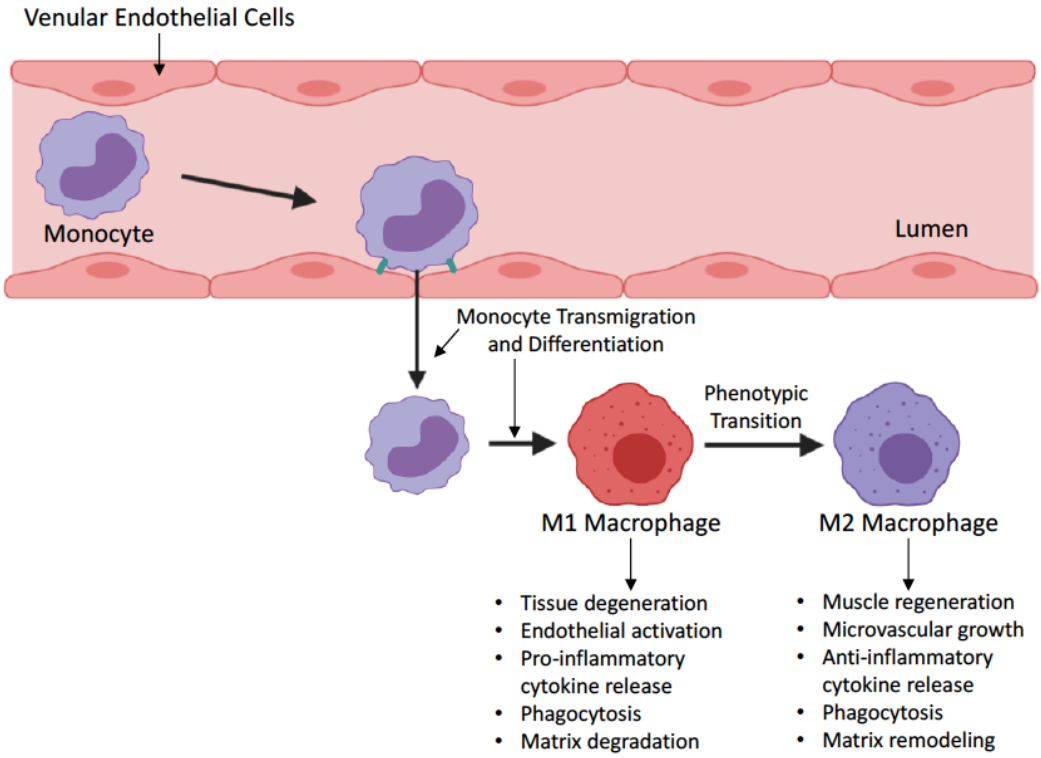

Figure 1.2: Monocyte and Macrophage Function During Inflammation. Schematic representing the recruitment, transmigration and differentiation of monocytes into macrophages in muscle tissue. Monocytes bind to EC surface proteins such as E-selectin or VCAM-1 (demarcated in green). A summary of the M1 to M2 phenotypic transition and their respective functions is also depicted. Created with BioRender.com.

1.5.2 Satellite Cells

ECs are also in close proximity to muscle stem cells, referred to as muscle satellite cells (MuSCs). Anatomically, MuSCs are sequestered between individual myofibers and the basal lamina (Wang

and Rudnicki, 2011). MuSCs are precursors to mature myocytes and are necessary for post-embryonic skeletal muscle development and regulating muscle regeneration following myotrauma. This has been demonstrated in models of MuSC depletion where muscle regeneration is severely impaired (Lepper et al., 2011; Sambasivan et al., 2011). In order for MuSCs to contribute to sufficient muscle regeneration, they need to undergo sequential phases of cellular differentiation that lead to myocyte maturation. Under basal conditions, MuSCs are maintained in the G_0 or quiescent phase of the cell cycle (Laumonier and Menetrey, 2016; Wang and Rudnicki, 2011). When the skeletal muscle is subjected to stressors such as mechanical forces, oxidative stress and inflammation, MuSCs become activated, re-enter the cell cycle and become myoblasts (Laumonier and Menetrey, 2016). Myoblasts will then proliferate and differentiate into myocytes, a more committed myogenic phenotype (Laumonier and Menetrey, 2016). Sequentially, myocytes will fuse together and elongate to form myotubes (Laumonier and Menetrey, 2016). After a period of growth, myotubes will then undergo terminal differentiation to become multinucleated myofibers. Lastly, myofibers will transition into a maturation phase where they will become mature myofibers (Yin et al., 2013). It is important to note that a small subset of activated MuSCs re-enter into quiescence through the process of self-renewal, which ensures the maintenance of a

stem cell pool in case of future injury (Dumont et al., 2015a, 2015b). A summary of the MuSC fate following injury is depicted in Figure 1.3.

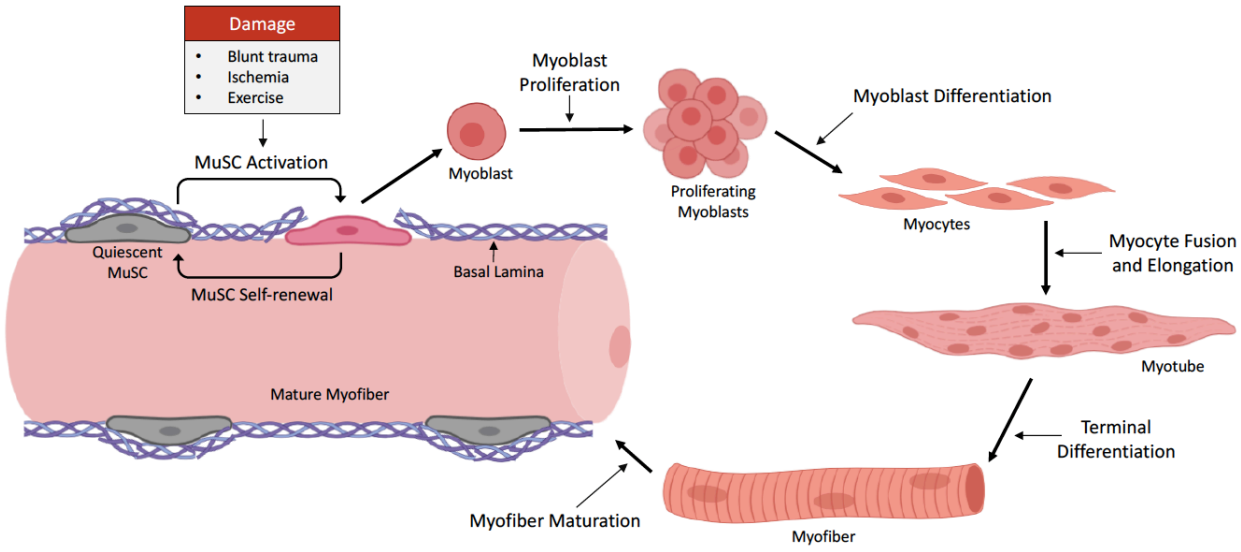

Figure 1.3: MuSC Fate Following Muscle Injury. Schematic representing the cellular fate of MuSCs following skeletal muscle injury. MuSC: muscle satellite cell. Created with BioRender.com.

1.5.3 Endothelial Interactions Between Immune Cells and Satellite Cells

The endothelium has the capacity to communicate and influence both immune cell and MuSC populations through paracrine or juxtacrine mechanisms (Christov et al., 2007) (Figure 1.1A, B). The first intimate relationship between the endothelium and immune cells occurs when plasma-located monocytes are attracted and bind to the luminal surface of the EC during inflammation (Figure 1.2). Monocytes bind to EC adhesion molecules such as E-selectin and VCAM-1, which facilitates the transmigration of monocytes within the regenerating tissue and the subsequent differentiation into macrophages (Muller, 2014; Yang et al., 2014). ECs are also known to release both pro-inflammatory (e. g. IL-6 and IL-1β) and anti-inflammatory (e.g. IL-10 and TGF-β) cytokines within the tissue microenvironment (Kofler et al., 2005). Through a paracrine mechanism, these factors can induce macrophages to assume an M1- or M2-like inflammatory phenotype. ECs can also release granulocyte growth factors that can stimulate immune cell

proliferation and differentiation (Mai et al., 2013). Overall, these processes exemplify the potential role of the EC in regulating inflammatory processes via EC-immune cell interactions. However, these processes have not been well investigated within the context of tissue injury and regeneration.

The MuSC-EC interaction has also been gaining attention within the field of skeletal muscle development and regeneration. Recent studies have shown that increased vascular density is positively correlated with satellite cell numbers (Matsakas et al., 2013). Additionally, transmission electron microscopy of skeletal muscle reveals that ECs and MuSCs are in close anatomical proximity (~2 μm distance) but they are physically separated by the basal laminae and basement membrane of both the capillary and the skeletal myofibers (Figure 1.1B). This suggests that paracrine, rather than juxtacrine, EC-MuSC interactions are possible when MuSCs are in a quiescent state (Christov et al., 2007; Yin et al., 2013). Paracrine signaling between ECs and MuSCs has been tested in vitro using cell cultures. Interestingly, EC-derived growth factors, IGF-1, HGF, PDGF-BB and VEGFA, promoted MuSC growth (Christov et al., 2007). It was also reported that MuSCs in vivo were considered pro-angiogenic because they are a source of VEGFA, highlighting the potential reciprocal relationship between ECs and MuSCs (Christov et al., 2007). Although theoretically possible, the way in which both cell types can influence their respective biological profiles has not been well established in vivo and during tissue regeneration.

1.6 Skeletal Muscle Regeneration Time-Line

By definition, skeletal muscle damage is a disruption of myofiber structure and function as a result of acute or chronic stressors such as intense bouts of exercise, blunt trauma and ischemia; all of which disrupt mechanical and/or metabolic processes (Warren et al., 2007; Laumonier and

Menetrey, 2016). The physiological processes that characterize muscle damage have been explored in severe injury models of blunt trauma and cardiotoxin injections. Myocyte membrane (sarcolemma) instability and redox imbalances occur in response to myofiber damage and can trigger a cascade of cell-intrinsic events that begin the processes of muscle regeneration (Laumonier and Menetrey, 2016; Tidball, 2011). Sarcolemma instability, as a result of trauma-induced micro-tearing, can lead to excessive calcium ion influx within the myocyte (Tidball, 2011). This enhances the activity of proteases, hydrolases and phospholipases, which trigger cell death signaling pathways and eventual necrosis of individual myofibers (Tidball, 2011). Calcium imbalances also lead to mitochondrial dysfunction and subsequent overproduction of ROS, which causes oxidative damage to proteins, DNA and lipids as well as triggers an apoptotic or pro-necrotic program (Pellegrino et al., 2011;Whitehead et al., 2006). Altogether, these molecular and cellular processes that define skeletal muscle damage will serve as a stimulus to initiate the regeneration time-line outlined in Figure 1.4 and Table 1.1.

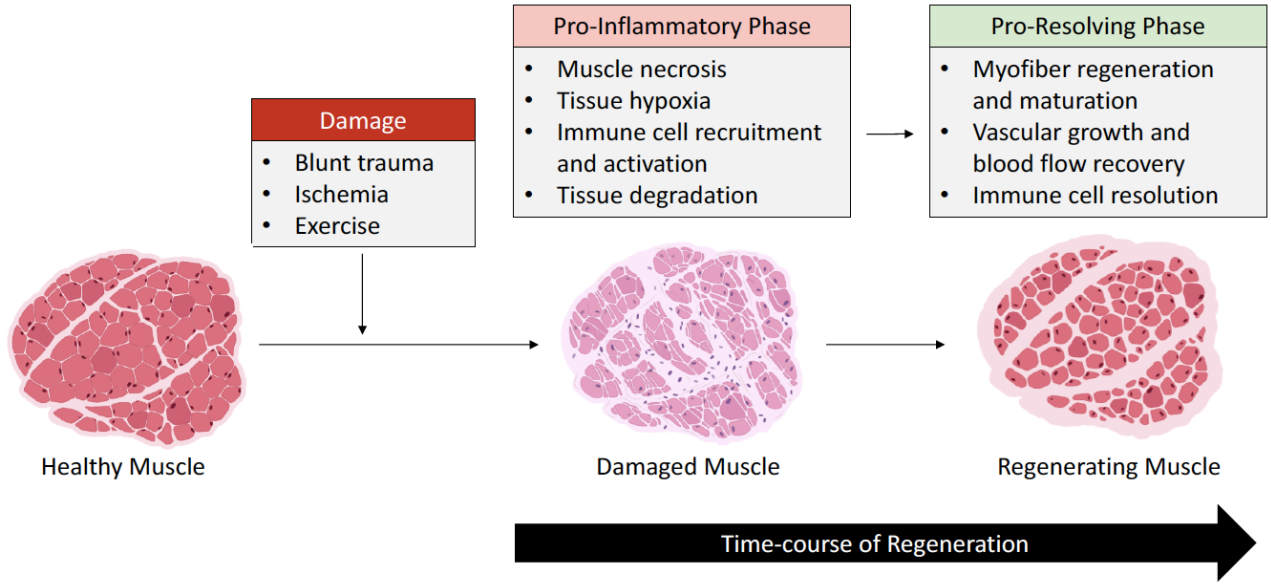

Figure 1.4: Key Events that Characterize the Time-course of Muscle Regeneration Following Damage. In general, muscle damage initiates the first phase of repair, pro-inflammation and degeneration. Subsequently, this transitions into the pro-resolving and regenerative phase where muscle tissue is restored. A more comprehensive summary is depicted in Table 1.1. Created with BioRender.com.

Table 1.1: **Skeletal Muscle Regeneration Time-Line:** Cellular and molecular events that occur following skeletal muscle damage.

Time Post Injury	Myogenic	Inflammatory	Vascular	Matrix Tissue
Hours to day 1	Myofibers produce and release growth factors and pro-inflammatory cytokines in response to oxidative stress.[1, 2, 3] *MuSC Activation*: MuSCs re-enter the cell-cycle and become proliferative myoblasts. Stimulated by NO and growth factors. [4]	Pro-inflammatory cytokines attract neutrophils into damaged muscle.[8, 9] Neutrophil levels peak and release M1-biased cytokines and free-radicals to degrade myocytes.[8, 10, 11]	Disruption in microvascular perfusion.[16] EC undergoes oxidative stress and activation. EC enhances expression of immune cell binding proteins and facilitate. [12, 13, 14, 15] Growth factors initiate angiogenic program [17]	ECM, basal laminae and basement membranes are degraded by MMPs.[18] ECM-sequestered growth factors are released.[19] MuSCs integrin-bound to basal lamina and ECM are detached and exposed to growth factors, cytokines and NO. Degraded laminins and fibronectin maintain MuSC activation.[18, 19]
Day 2 to 4	*Myoblast Proliferation*: myoblasts proliferate and migrate to damaged myofibers.[7, 8]	M1 macrophage levels peak and further degrade myocytes, phagocytose debris and dying neutrophils. Neutrophil levels decline.[8]		
Day 4 to 7	*Myoblast Differentiation*: myoblasts exit cell-cycle and differentiate into myocytes. Enhance production of myogenic structural and functional genes such as actin and myosin. Myocytes fuse and elongate into multinucleated myotubes.[2, 4, 22, 23]	M1 macrophages shift into an M2-like phenotype, mediated by macrophage phagocytosis of cellular debris and M2 cytokines. M2 macrophages are the predominant cell-type. They suppress M1 tissue degradation, increase immune cell resolution and release regenerative growth factors. Promote myogenesis, vascular growth and tissue remodeling.[8]	Restoration of capillary perfusion.[16]	Growth factors and cytokines stimulate fibroblasts to synthesize and deposit collagen and laminin within the ECM.[4, 20] FAP cells contribute to myogenic program, accumulate adipocytes in ECM.[21]
Day 7 to 14	*Myotube Terminal Differentiation*: myotubes become more committed, fuse to damaged myofibers and undergo maturation and growth.[4]	Immune cell populations diminish.[8]	Growth factors and cytokines (from macrophages or myocytes) stimulate angiogenesis.[24] Enhanced growth detected as early as day 7.[24] Vasomotor tone, EC-dependent vasodilation and adrenergic vasoconstriction restored.[16]	ECM accumulation and restructuring. Growth factors and MuSCs re-sequestered via integrin binding. Fibroblast, FAPs and adipocyte levels diminish.[8]
Day 14 to 21	Myofibers undergo hypertrophy and increase cross-sectional area.[2, 4]		Vasomotor control restored.[16]	

Table 1.1 Reference Legend: [1](Clarke and Feeback, 1996), [2](Laumonier and Menetrey, 2016), [3](Yang et al., 2014), [4](Yin et al., 2013), [5](Wieteska-Skrzeczyńska et al., 2011), [6](Mourkioti and Rosenthal, 2005), [7](Yablonka-Reuveni and Rivera, 1997), [8](Tidball, 2011), [9](Kozakowska et al., 2015), [10](Nguyen et al., 2005) [11](Toumi et al., 2006a), [12](Liu et al., 2017), [13](Monaco and Paleolog, 2004), [14] (Pober and Sessa, 2015), [15](Wittchen, 2009), [16](Fernando et al., 2019), [17](Hardy et al., 2016), [18](Thomas et al., 2015) [19](Dumont et al., 2015a), [20](Sarkar et al., 2004), [21](Joe et al., 2010), [22](Guo et al., 1995), [23](Penn et al., 2004), [24](Ochoa et al., 2007).

1.7 Epidemiology and Clinical Implications of Peripheral Arterial Disease

1.7.1 Epidemiology of PAD

Peripheral artery disease (PAD) is a common manifestation of atherosclerosis and is characterized by an accumulation of plaque within the arteries supplying blood to the lower extremities (Lovell et al., 2009). Distal to the occlusion, the ischemic muscle is deprived of oxygen and nutrients, lowering viable tissue health. Therefore, PAD is a vascular complication that is associated with severe skeletal muscle damage. Epidemiological data suggests that over 200 million people worldwide are diagnosed with PAD with a 5-year mortality rate as high as 30% (Lovell et al., 2009). Reports have also indicated that the incidence of PAD increase sharply with age and is predominantly diagnosed in males (Cornejo del Río et al., 2017). Additionally, cardiovascular and metabolic complications such as diabetes, obesity and dyslipidemia are strong predictors of PAD diagnosis and can lead to the exacerbation of negative disease outcomes (Hicks et al., 2018).

1.7.2 Clinical Manifestations of PAD and Ischemic Muscle Damage

Rather than being a transient event, the cellular processes that lead to vascular occlusion can take years before symptoms can be detected. As a result, patients are often undiagnosed or undertreated due to the relatively asymptomatic nature of the condition (McDermott, 2015). Therefore, PAD has been characterized by a spectrum of disease severities ranging from asymptomatic to symptomatic stages. The early stages of PAD are marked by small reductions of blood flow to the distal limb and minimal muscle damage; therefore, no symptoms are detected. As the disease

progresses, arterial blood flow is further diminished and myofibers receive less oxygen and nutrients to maintain adequate health (McDermott, 2015; Rutherford et al., 1997). This can lead to intermittent claudication which is the onset of leg pain while walking and is usually associated with abnormal gait (McDermott, 2015; Rutherford et al., 1997). The severity of intermittent claudication can progress from mild to severe and is correlated with the extent of ischemic muscle damage. In more chronic stages of PAD, patients may transition from intermittent claudication to resting leg pain, which is a sign of more progressive muscle degeneration (Norgren et al., 2007; Rutherford et al., 1997). The most severe form of PAD is known as critical limb ischemia (CLI) and presents as distal limb necrosis and gangrene which can suddenly occur from plaque rupture or thrombosis of the artery. As a result of limb necrosis, CLI can lead to the requirement for limb amputation (Norgren et al., 2007; Rutherford et al., 1997). PAD patients with intermittent claudication have difficulty with every day activities that can diminish their overall quality of life and have reduced life expectancy (Norgren et al., 2007; Carter et al., 1989). However, individuals with CLI have a greater risk for mortality (Schieber et al., 2017).

1.7.3 Surgical Therapeutics and Persistent Ischemic Myopathy

The most common therapeutic procedure for PAD treatment are surgical interventions that aim to restore blood flow to the lower limb. These include arterial bypass or angioplasty that facilitate the restoration of bulk blood flow to distal muscle groups (Rice and Lumsden, 2006). Although these techniques solve the big problem – lack of blood flow – PAD patients still experience persistent muscle degeneration (Lundgren et al., 1989; Owens et al., 2008; Robinson et al., 2011). This is because current surgical interventions neglect the fact that PAD pathology is characterized by impaired microvascular remodeling and perfusion, as well as persistent tissue-level inflammation

and myofiber degeneration (Jones et al., 2012; Pipinos et al., 2007). The negative clinical manifestations of PAD point towards impairments in how the cells of the skeletal muscle micro niche respond to ischemic damage. Unfortunately, a comprehensive understanding of how this occurs has not been established. Therefore, it is crucial to investigate the cellular and molecular mediators of post-ischemic muscle regeneration and potential therapeutic targets that can aid in better management of PAD.

The extent of muscle recovery following arterial occlusion depends on sufficient micro and macro-level vascular remodeling. Collateral arteries (deriving upstream of the blockage) can undergo arteriogenesis which is when the vessel expands in diameter (Figure 1.5). Arteriogenesis occurs in non-ischemic regions of the lower limb and can enhance macro-level blood flow to distal muscle groups (Heuslein et al., 2015). Microvascular remodeling such as arteriolargenesis and angiogenesis occur within the ischemic skeletal muscle (Figure 1.5). Arteriolargenesis is when capillaries transition into arterioles and can increase the distribution of micro-level perfusion within the regenerating muscle (Hansen-Smith et al., 2001). Lastly, angiogenesis within the ischemic muscle is the expansion of the capillary bed and can contribute to increased diffusion of oxygen and nutrients to regenerating myofibers (Figure 1.5). Further detail on these processes will be discussed in section 1.9.2.

Ischemic myopathies may in part be attributed to impaired microvascular remodeling that occurs in the ischemic muscle of PAD patients. For example, compared to normal individuals, PAD patients have reduced capillary density and capillary to fiber ratio in skeletal muscle biopsies and often have impaired perfusion (Jones et al., 2012; Robbins et al., 2011). This can have a profound

effect on skeletal muscle regeneration as capillaries control the diffusion of nutrients and oxygen to regenerating myofibers. More specifically, it has been shown that amino acids and insulin stimulate a cascade of cell signaling events (via mTOR) that enhances myofiber protein synthesis, which effectively promotes the maturation of the cell (Timmerman et al., 2010). Additionally, plasma-derived oxygen delivery can overcome the hypoxic challenge that ischemic myofibers are subjected to. This can alleviate myocyte mitochondrial stress and restore redox homeostasis. The lack of a capillary network can also impair the removal of cellular debris and metabolic waste, which can accumulate in post-ischemic muscle and exacerbate tissue damage (Pober and Sessa, 2015). Unfortunately, the specific reasons for these outcomes has not been well established. Therefore, a more comprehensive assessment of the cellular and molecular mediators of ischemic muscle regeneration needs to be investigated.

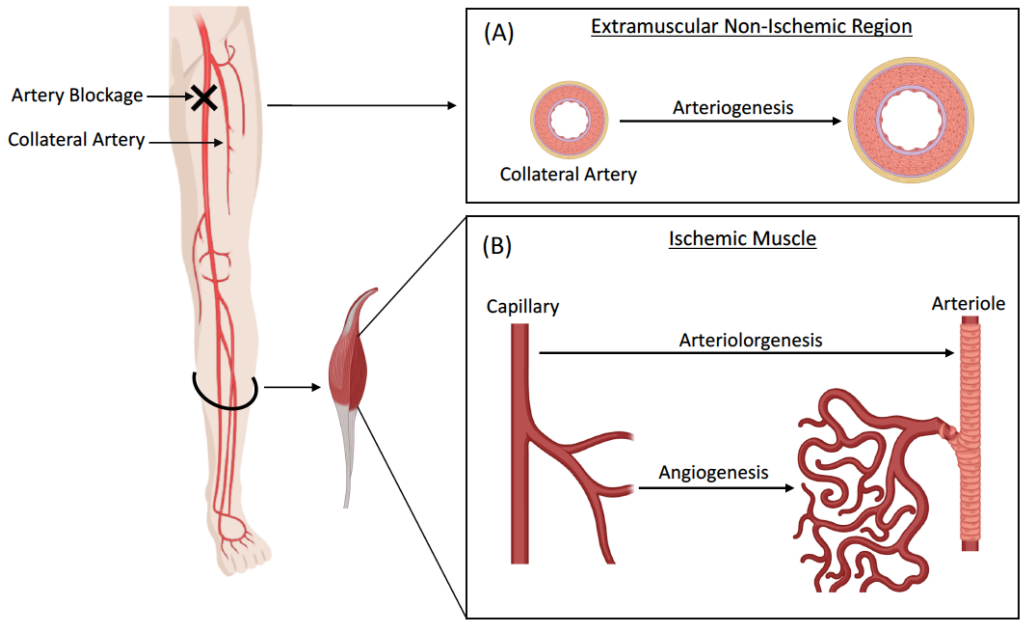

Figure 1.5: Vascular Remodeling Following Arterial Occlusion. (A) Collateral arteries undergo arteriogenesis (e. g. vessel enlargement) in non-ischemic regions. (B) Within ischemic muscle, arteriolargenesis occurs when capillaries transition into arterioles and angiogenesis is capillary expansion. Created with BioRender.com.

1.8 Mouse Models of Critical Limb Ischemia

1.8.1 Surgical Approaches to Generate Hind Limb Ischemia

Mouse models of hind limb ischemia have been used in pre-clinical studies to investigate the cellular and molecular aspects of PAD and to identify therapeutic interventions. This allows for a more comprehensive and in-depth assessment of disease etiology which can then be used in translational research. However, these models do not emulate the multifaceted nature of PAD and are more accurately referred to as mouse models of CLI. Mice are resistant to developing atherosclerotic plaque, therefore, surgical interventions to induce CLI are required. The femoral artery is a common target for surgically-induced CLI because it is large, easily accessible and supplies blood to distal skeletal muscles (e. g. gastrocnemius and extensor digitorum longus) (Aref et al., 2019).

Two common surgical procedures are excision and ligation of the femoral artery. Femoral excision is the removal of the femoral artery and supporting collateral vessels, resulting in 90% reduction in limb blood flow (Couffinhal et al., 1998). Additionally, collateral vessel formation is impaired, prolonging blood flow recovery and exacerbating the severity of ischemia (Yu and Dardik, 2018; Aref et al., 2019). However, vessel excision may not accurately represent clinical CLI and is not a good model for assessing arteriogenesis (Aref et al., 2019). On the contrary, femoral artery ligation is the use of a suture to tie a portion of the artery, preventing blood flow to distal tissues. The severity of ischemia can differ depending on the location of the ligation along the femoral artery. Moderate ischemia can be induced by ligating the femoral artery distal to the epigastric artery which shunts blood to supporting collateral vessels (Aref et al., 2019; Roudier et al., 2013). This reduces approximately 50% of resting blood flow to the lower limb. However, this approach

may not lead to robust muscle damage or inflammation that are similarly observed in clinical CLI; as it is more a model of intermittent claudication. Therefore, severe femoral artery ligation has been developed as a better model to mimic the severity of CLI. In this technique, the femoral artery is ligated immediately distal to its exit from the abdominal wall, preventing blood to be shunted to supporting collateral vessels. This approach is a more accurate representation of CLI as it results in 85-90% reduction of blood flow to the lower limb, keeps major arterial branches intact and induces sufficient skeletal muscle damage (Aref et al., 2019).

1.9 Pathophysiology of Ischemic Muscle Damage

Ischemia elicits muscle damage as a result of both transient and prolonged reductions of blood perfusion. Impairments of blood flow lead to diminished oxygen and nutrient delivery to individual myofibers and results in oxidative and nutrient-deprived stress and eventual death of myocytes (Krishna et al., 2015). Damaged post-ischemic myofibers are necrotic, apoptotic, atrophied (reduced cross-sectional area) and have centrally located nuclei (Koutakis et al., 2015; Pipinos et al., 2007). In conjunction, histological assessments reveal increases in collagen deposition within the extracellular compartments of the muscle (Pipinos et al., 2007). At a micro-scale, electron microscopy of ischemic myofibrils reveals abnormal sarcomere structures, impaired cross-bridge formation and swollen mitochondria with lipid vacuoles (Pipinos et al., 2007). Altogether, these observations can explain the severe reduction in muscle function associated with ischemic myopathy in PAD patients. However, the exact reasons for these pathological outcomes are not well established. By assessing mouse models of CLI at multiple time-points, the phases of post-ischemic muscle recovery and the corresponding regenerative processes can be better understood. Ultimately, we can learn from muscle regeneration time-lines well established in injury (e. g. CTX and blunt trauma) models (discussed in Table 1.1) and apply these concepts to hind limb ischemia.

Presently, hind limb ischemia models use time-lines (day 4, 7, 11 and 14) that assess blood flow recovery and vascular remodeling, however, with less emphasis on the myogenic and inflammatory aspects of regeneration (Brenes et al., 2012). Therefore, I will now highlight what is known about the recovery time-line associated with severe muscle ischemia as well as the similarities and differences in comparison to other muscle injury models.

1.9.1 Molecular and Cellular Stages of Ischemic Myopathy

Myogenic Response: Acute and chronic stages of muscle ischemia share similar features observed during muscle trauma. For example, mitochondrial respiration and membrane potential are impaired at 5 hours of ischemia (Brandão et al., 2003). This coincides with elevated reliance on glycolytic metabolism as intracellular lactate levels rise within 6 hours of ischemia (Paradis et al., 2016). In chronic stages of ischemia (12 weeks), impaired mitochondrial activity drastically depletes intracellular ATP reserve (Pipinos et al., 2008). This can lead to excess accumulation of cytosolic calcium and, in turn, cause cellular damage by reducing myocyte membrane integrity and eliciting a pro-inflammatory and apoptotic/necrotic program (Paradis et al., 2016).

***Vascular Response*:** Vascular integrity and morphology is known to change in response to hind limb ischemia. For example, rats subjected to acute hind limb ischemia experienced enhanced vascular permeability (detected by protein leakage) as early as 1-3 hours of ischemia (Sternbergh, 1992). Additionally, electron microscopy revealed ECs initially appeared intact with normal vacuole structures at 1 hour, however, this was distorted at 2 hours of ischemia (Sternbergh, 1992). This also included intracellular edema and larger vacuoles extending into the vessel lumen (Sternbergh, 1992). Reports also demonstrate that ischemia degenerates the outer glycocalyx layer of the EC which triggers EC pro-inflammatory signaling (Annecke et al., 2011). Hypoxia can also

induce HIF-1α- and/or NFκβ-mediated EC pro-inflammation by increasing intracellular ROS and the expression of immune-cell binding proteins ICAM-1, E-selectin and VCAM-1 (Liang et al., 2019; Ben-Shoshan et al., 2009). These activated ECs will then mediate subsequent inflammatory processes that are required for ischemic muscle recovery.

***Inflammatory Response*:** EC pro-inflammation mediates immune cell recruitment and transmigration into ischemic muscle to initiate the inflammatory cascade. *In vivo*, it was reported that the expression of E-selectin on microvessels from ischemic skeletal muscle peaked at 6-12 hours of ischemia (Oh et al., 2007). At day 3 of ischemia, leukocyte rolling and adhesion to post-capillary venules was enhanced which corresponded with elevated EC ICAM-1 expression (Anderson et al., 2006). This demonstrates that EC activation is a major reason why peak immune cell infiltration occurs within 3 days of ischemia (Paoni et al., 2002). Once neutrophils and/or M1 macrophages are present within the ischemic muscle, they produce and secrete ROS which further stimulates pro-inflammatory and apoptotic signaling pathways (Tidball and Villalta, 2010).

1.9.2 Angiogenesis and Arteriogenesis in Ischemic Hindlimbs

Angiogenesis: Skeletal muscle ischemia elicits an angiogenic time-line that closely parallels with muscle recovery (Figure 1.5). In general, capillary numbers tend to increase from the early to late stages of ischemia and then plateau when muscle recovery is complete (Couffinhal et al., 1998). Multiple studies have highlighted the role of numerous cells, growth factors and cytokines that can modulate capillary growth in animal models of hind limb ischemia which is discussed below.

In a model of severe hind limb ischemia, VEGFA mRNA and protein levels increased in regenerating myofibers, ECs and infiltrating immune cells (Couffinhal et al., 1998). Increased

presence of VEGFA was also associated with elevated CD, suggesting these cell-types may be responsible for VEGFA-induced angiogenesis. Interestingly, blocking VEGFA using a neutralizing VEGFA antibody led to less of an increase in CD in 14-day ischemic muscle (Couffinhal et al., 1998). However, the presence of VEGFA alone may not be sufficient to elicit capillary growth. This was demonstrated in diabetic mice where there was an observed increase in VEGFA protein in 3-day ischemic muscle, however, angiogenesis was impaired as a result of reduced VEGFA signaling (e. g. lower pAKT levels) (Hazarika Surovi et al., 2007). Other factors, independent of VEGFA, can also serve a pro-angiogenic role in the ischemic muscle. For example, mice with a homozygous deletion of eNOS ($eNOS^{-/-}$) showed impaired skeletal muscle CD at days 14, 21 and 35 of ischemia which coincided with reductions in post-ischemic blood flow recovery (Murohara et al., 1998). The ischemic muscle is also comprised of pro-angiogenic M2-like cytokines (released from macrophages or myofibers) that play a crucial role in post-ischemic capillary growth. This was exemplified in mice lacking M2 cytokine, IL-19, which resulted in inpaired angiogenesis at day 15 of ischemia. Mechanistically, it was demonstrated that IL-19 stimulates M2-macrophage differentiation, enhancing macrophage production and release of VEGFA, as well as reducing the production of anti-angiogenic cytokine IL-12 (Richards et al., 2015). Altogether, these studies exemplify how multiple cell-types, growth factors and cytokines within the regenerating niche can contribute to angiogenesis in ischemic muscle.

Arteriogenesis: Blood flow recovery following hind limb ischemia is attributed to collateral vessel growth which is the expansion (e. g. vessel enlargement) of pre-existing arteries in the non-ischemic regions of the hind limb (Figure 1.5). We and others have established a time-course of blood flow recovery at multiple days following the induction of severe hind limb ischemia. In

general, mice experience a gradual increase in hind limb blood flow which eventually plateaus in the late stages of ischemia (Nwadozi et al., 2020; Couffinhal et al., 1998). Hemodynamics play a crucial role in the enlargement of collateral vessels following the occlusion of an artery. It is known that an upstream occlusion leads to a change in the pressure gradient that shunts blood flow through supporting collateral vessels which effectively increases their shear stress. Ultimately, this stimulus results in an increase in collateral vessel diameter and is a major contributor to blood flow recovery. In fact, this is why mice subjected to hind limb ischemia appear to have a 2-fold increase in collateral diameters over a 21-day period (Scholz et al., 2002).

The magnitude of blood flow recovery can also vary depending on the number and location of pre-existing collateral arteries as well as new collateral vessel formation. These parameters are dependent on inherent genetics (e. g. heterogeneity in humans or mouse strains) as well as the response of numerous cell-types, growth factors and cytokines within the regenerating muscle. Interestingly, it has been shown that VEGFA is not a major growth factor responsible for ischemia-induced arteriogenesis. In fact, the VEGFA-family ligand, placenta growth factor (PLGF), is a more prominent arteriogenic growth factor. For example, following the induction of severe hind limb ischemia, PLGF knockout mice had impaired collateral vessel growth (Carmeliet et al., 2001). It is also established that arteriogenesis is highly dependent on the actions of macrophages/monocytes. In general, mice genetically deficient in monocytes (90% reduction in circulating levels) developed less collateral vessels in response to hind limb ischemia and this coincided with impaired blood flow recovery (Bergmann et al., 2006). Additionally, Bruce et al. demonstrated that macrophages and monocytes reside in close proximity to the vascular endothelium of arterioles and this aggregation was enhanced during arteriogenesis in ischemic

muscle (Bruce et al., 2014). Takeda et. al. further elucidated that M2 macrophages are necessary for ischemic arteriogenesis. For example, prolyl hydroxylase domain-containing protein 2 haplodeficient ($Phd2^{-/+}$) mice with a more enriched expansion of tissue-resident M2 macrophages experienced extensive collateral vessel growth in ischemic muscle compared to their wildtype counterparts. This also correlated with enhanced lower limb blood perfusion and muscle regeneration (Takeda et al., 2011). Lastly, Krishnasamy and colleagues proposed that during ischemia, vascular ECs can differentiate circulating monocytes into M2 macrophages through angiocrine Notch-Dll1 signaling. In turn, this promoted enhanced arteriole branching, arteriole enlargement as well as greater blood flow and muscle recovery in ischemic hind limbs (Krishnasamy et al., 2017).

1.9.2 Therapeutic Angiogenesis

Re-establishing this vascular network is a major limiting factor in post-ischemic muscle regeneration. This suggests that CLI is a result of a dysregulated balance between pro- and anti-angiogenic factors which tends to favor capillary regression rather than the desirable capillary growth. To circumvent the overall lack of post-ischemic vascular growth, researchers have tried to enhance this process in both human and mouse models of CLI by using growth factor therapy or cell-based therapy (Annex, 2013). Growth factor therapy is the delivery of pro-angiogenic proteins whereas cell-based therapy is administering cells that can release growth factors through paracrine mechanisms (Annex, 2013). Pre-clinical models of hind limb ischemia have shown promise when using recombinant administration of VEGFA, reporting improved blood flow recovery and vascular remodeling (Takeshita et al., 1996). However, these findings have not been recapitulated in clinical trials as reports demonstrate no benefits in VEGFA and other growth factor

treatments in ischemic limbs of PAD patients (Dragneva et al., 2013). It was also reported that the administration of these growth factors can lead to vascular instability and leakage (Dragneva et al., 2013; Petrak et al., 2019).

1.10 Endothelial FoxO Proteins and Post-Ischemic Muscle Recovery

The lack of efficacy in current therapeutic angiogenic strategies indicates the need to investigate the role of other factors that can modulate vascular growth. Additionally, a more effective therapeutic can also target other aspects of post-ischemic muscle recovery such as inflammation and myofiber regeneration. Therefore, we chose to investigate the other side of the angiogenic spectrum, elucidating the role of anti-angiogenic factors in hind limb ischemia as well as their potential to influence inflammation and myogenesis.

1.10.1 Endothelial FoxO Proteins and their Therapeutic Potential

Microvascular Role: Our lab has previously reported an impairment of angiogenesis in the ischemic muscle of mice subjected to moderate ischemia. Despite the muscle being enriched with a pro-angiogenic environment – with observed elevation of VEGFA – vascular growth was impaired, suggesting a potential role in the concomitant upregulation of anti-angiogenic factors (Roudier et al., 2013). Interestingly, endothelial FoxO1 protein was increased within the ischemic muscle of PAD patients and mice, suggesting a potential skew towards an anti-angiogenic phenotype (Roudier et al., 2013). It was also demonstrated that adult mice with an induced EC-directed depletion of FoxO1, 3 and 4 showed greater blood flow recovery and angiogenesis (higher capillary to fiber ratio) following mild hind limb ischemia (Roudier et al., 2013). Within the same study, it was also established that FoxO1 is a direct transcriptional regulator of the anti-angiogenic factor THBS1 within endothelial cells. Ultimately, it was determined that a possible mechanism

for impaired angiogenesis was through the activation of the endothelial FoxO1-THBS1 axis. The singular role of FoxO3 was also assessed by Potente et al. (Potente et al., 2005). More specifically, mice with a global embryonic deletion of FoxO3 ($FoxO3^{-/-}$) showed enhanced blood flow recovery and capillary density compared to FoxO3-expressing ($FoxO3^{+/+}$) mice. Interestingly, it was also established that the number of arterioles (actin+ vessels) was elevated in the ischemic muscle of $FoxO3^{-/-}$ compared to $FoxO3^{+/+}$ mice (Potente et al., 2005). This suggests that FoxO3 can negatively regulate post-ischemic capillary and arteriogenic remodeling. However, since this study used a global deletion of FoxO3, it is not clear which cell-type was responsible for these outcomes.

Myogenic Role: Beyond influencing angiogenesis and arteriogenesis, it is possible that EC-FoxO proteins can modulate the post-ischemic myogenic program. It is known that both EC-FoxO1 and 3 negatively regulate eNOS transcription and, in turn, can reduce EC NO bioavailability (Potente et al., 2005). Interestingly, NO (i. e. from ECs) can promote satellite cell activation (Wozniak et al., 2005). Therefore, EC-FoxO proteins could potentially suppress satellite cell activation by reducing EC-derived NO. EC-FoxO protein influence on EC metabolism may also serve as a potential link in the EC-MuSC crosstalk during muscle regeneration. EC-FoxO proteins suppress EC metabolism. For example, in EC-FoxO-depleted mice subjected to a high-fat diet (i. e. a growth stimulus), EC glycolysis is enhanced which led to the production and release of lactate from the EC. Interestingly, research has shown that lactate is a crucial regulator of the myogenic program as it can activate MuSCs and promote the differentiation and maturation of myotubes (Ohno et al., 2019). This suggests that EC-FoxO proteins can potentially suppress the pro-myogenic functions of lactate via its metabolic regulation within a cytokine- and growth-factor enriched environment of ischemic muscle.

Inflammatory Role: In numerous cell-types, FoxO proteins have been reported to enhance the expression of pro-inflammatory cytokines such as IL-1β and IL-6, which can contribute to an M1-biased microenvironment (Ito et al., 2009; Wang et al., 2014b). Within the context of ischemia, this can suppresses pro-regeneration and immune cell resolution (Wang et al., 2014a). We have previously investigated how EC FoxO proteins can mediate the inflammatory profile of skeletal muscle. Specifically, high-fat fed mice with an endothelial-specific depletion of FoxO1, 3a and 4 showed an enhanced M2-like expression profile within plantaris muscle. M1-related genes such as *Il6* and *Il1b* were downregulated, whereas M2-related genes such as *Tgfb*, *Il13* and *Mrc1* were upregulated (Nwadozi et al., 2016). This phenotype may have a profound effect on muscle resident FAP cells, fibroblasts and macrophages within the context of ischemia. In an M2 micro-environment, the pro-adipogenic or fibrotic functions of FAPs can be mitigated as well as the pro-fibrotic functions of fibroblasts. Additionally, an M2 cytokine environment can stimulate pro-regenerative properties of macrophages which can assist in myofiber and vascular regeneration.

Although EC-FoxO proteins can theoretically influence the myogenic, inflammatory and vascular responses to hind limb ischemia, these effects have not been well investigated. Taking this one step further, the distinct or overlapping roles of EC-FoxO1 and 3 have not been established.

1.11 Rational for Thesis, Hypothesis and Objectives

Endothelial cells play a central role in orchestrating the response to muscle injury as it can influence myogenic, inflammatory and vascular processes. However, this potential has not been established within the context of post-ischemic muscle regeneration. By altering the biological profile of the EC in ischemic muscle, we can more concretely identify the ECs specific role in the recovery time-line. Therefore, we targeted endothelial-specific FoxO proteins because of their

profound roles in mediating vascular homeostasis, pro-inflammatory potential as well as plausible anti-myogenic influences on ischemic muscle (discussed in section 2.1).

Our previous study identified that EC-FoxO proteins restrain skeletal muscle angiogenesis following moderate hind-limb ischemia (Roudier et al., 2013). However, there was an insufficient threshold of blood flow reduction to elicit severe muscle damage. Therefore, it remains unclear how EC-FoxO proteins can modulate microvascular remodeling, inflammation and myofiber regeneration within the context of severe muscle ischemia. Our previous study was also subjected to various limitations:

1. Using an inducible Cre recombinase under the transcriptional control of an Mx1 promotor is non-specific to ECs, as it can be activated in hematopoetic derived immune cell populations (Velasco-Hernandez et al., 2016;Sengupta et al., 2012). FoxO isoforms can directly regulate T cell and dendritic cell biology by altering their quiescence and activation, therefore, this may pose as a confounding effect when assessing muscle regeneration (Hedrick et al., 2012).
2. Depleting all three EC-FoxO isoforms limits the ability to distinguish the singular and synergistic functions of EC-FoxO1 and 3 in muscle ischemia.

My thesis project aims to circumvent these limitations as follows:

1. Implementing a more EC-specific depletion of FoxO isoforms using a Tamoxifen-inducible Cre recombinase (iCreER^{T2}) under the transcriptional control of the PDGFB promoter (*Pdgfb-iCre/ER^{T2}*) (Claxton et al., 2008).

1. Segregating the singular and synergistic roles of EC-FoxO1 and 3 by using mice with a single (EC-FoxO1 KD) and double (EC-FoxO1,3 KD) depletion of EC-FoxO protein and compare these to Controls (EC-FoxO1,3-expressing).

1.11.1 Study Objectives

1. To test whether the depletion of EC-FoxO1 and EC-FoxO1,3 alter the angiogenic and arteriogenic responses to severe muscle ischemia.

2. To establish if EC-FoxO1 and FoxO1,3 depletion exert redundant or additive effects of inflammation, myocyte regeneration and tissue remodeling in response to severe hind limb ischemia.

1.11.2 Hypothesis

EC-FoxO depletion will enhance blood vessel growth and blood flow recovery, inflammatory resolution and muscle regeneration within the ischemic muscle. However, the combined depletion of EC-FoxO1 and 3 will exert a greater influence than EC-FoxO1 alone.

Chapter 2: Methods

2.1 Ethical Approval

Animal studies were approved by the York University Committee on Animal Care (#2017-19R3, #2017-20R3).

2.2 Endothelial Cell Specific Depletion of FoxO1 and FoxO3

Male mice with flanked loxp sites of FoxO1 (*Foxo1*$^{f/f}$) or both FoxO1 and 3 (*FoxO1,3*$^{f/f}$) were generated by the outbreeding of FoxO1,3,4$^{f/f}$ mice with wild-type FVB/n mice (Paik et al., 2007). Genotyping of offspring was then used to ensure the floxed allele(s) were homozygous. To target an endothelial-specific depletion of FoxO1 and FoxO3, offspring were bred with Pdgfb-iCreER^{T2} mice (C57Bl/6 background). These mice express tamoxifen-inducible Cre recombinase (iCreER^{T2}) which is under the transcriptional control of Pdgfb, an endothelial specific gene (Claxton et al., 2008). *Pdgfb-iCre/ER*^{T2} mice were back-bred to the *Foxo1*$^{f/f}$ or *Foxo1,3*$^{f/f}$ founders, generating *Pdgfb-iCre/ER*^{T2}*:Foxo1*$^{f/f}$, *Pdgfb-iCre/ER*^{T2}*:Foxo1,3*$^{f/f}$, and control Cre- littermates (*Foxo1,3*$^{f/f}$ or *Foxo1,3*$^{f/f}$ mice), on a mixed FVB/n/C57Bl6/J background. Five consecutive injections of tamoxifen (300µg) were administered to each mouse at 5 weeks of age, which induced the deletion in any *Pdgfb-iCre/ER*$^{T2+}$ mouse. This resulted in an EC specific deletion of FoxO1 (EC-FoxO1 KD) and double deletion of EC-FoxO1 and EC-FoxO3 (EC-FoxO1,3 KD). Additionally, tamoxifen injections did not induce the deletion of EC-FoxO proteins in Cre- mice, thus maintaining the expression of EC-FoxO1- and EC-FoxO1,3 (Control). Our previous work confirmed that this method induces approximately 80% reduction in EC-FoxO proteins with no compensatory upregulation of EC-FoxO3 in EC-FoxO1-depleted animals (Rudnicki et al., 2018).

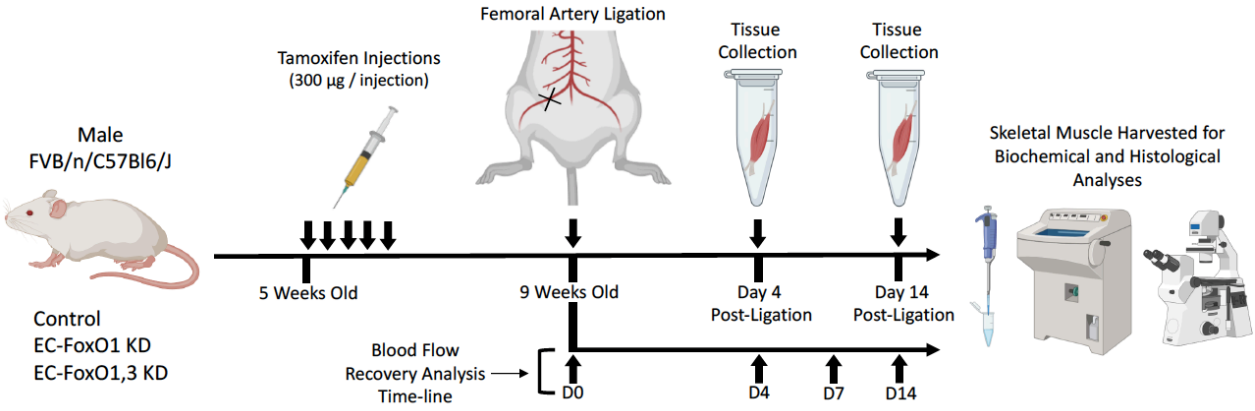

Figure 2.1: Schematic of study time-line. X demarcates the location of the femoral artery ligation surgery. D0, D4, D7 and D14 represent days after surgery at which blood flow measurements were taken.

2.3 Mouse Model of Femoral Artery Ligation

Male mice at 9 weeks of age underwent femoral artery ligation surgery (Nwadozi et al., 2020). Hair was removed using hair removal cream to expose the skin of the lower legs. Mice were anesthetized using isoflurane. At the right medial thigh, an incision was made to expose and isolate the common femoral artery immediately distal to its exit from the abdominal wall. A 6-0 silk suture was used to tie the common femoral artery, resulting in approximately 90% reduction of blood flow to the distal portion of the right limb. The incision of the medial thigh was then closed using a 5-0 silk suture and mice were postoperatively administered buprenorphine (0.05 mg/kg) for pain relief and ampicillin (20mg/kg in drinking water) to control for infection. The left limb did not undergo femoral artery ligation surgery and was used as a comparative control (non-ischemic) as blood flow was undisturbed in the distal portion of the leg.

2.4 Hind Limb Blood Flow Recovery Analysis

Hind limb blood flow was measured using non-invasive laser Doppler (Perimed) imaging. Mice were anesthetized with isoflurane and body temperature was maintained using a heating pad. Blood perfusion was measured before and immediately after common femoral artery ligation

surgery to assess relative blood flow at baseline and post-ligation, respectively. Perfusion recovery was then measured at day 4, 7, 11 and 14 following the ligation surgery. At all time-points, the distance of the laser Doppler to the animal limbs was held constant (20 cm). The dorsal side of the paws and the front of the legs in both ligated and non-ligated limbs were imaged for blood perfusion quantification. Briefly, laser Doppler images were traced around the perimeter of the paw or leg where a blood flow signal was detected. The average pixel intensity of the traced signal was then used to assess relative blood perfusion. The quantification was presented as a ratio of perfusion area of the ligated leg to that of the non-ligated leg.

2.5 Skeletal Muscle Tissue Collection

Skeletal muscle was collected from mice subjected to 4 and 14 days of ischemia. Plantaris, soleus and gastrocnemius muscles were excised from both legs and snap frozen in liquid nitrogen to be used for protein or RNA analysis. Extensor digitorum longus (EDL) muscle was extracted, embedded in optimal cutting temperature compound (OCT), frozen in liquid nitrogen-cooled isopentane and then submerged in liquid nitrogen to preserve for histological analysis.

2.6 Histological Analyses

2.6.1 Cryomicrotome and Muscle Mounting

EDL muscles preserved for histology were sectioned from the mid-belly and 10 μm transverse-plane sections were mounted on a histology slide. A Zeiss inverted microscope with a cooled CCD camera and Metamorph software was used to capture two to three representative images of each muscle section taken at the 10X objective. This was used in the following microvascular and myofiber assessments.

2.6.2 Microvascular Assessments

14-day post-ligation EDL muscle cryosections were co-stained with Griffonia simplicifolia lectin I (GSL)-FITC (#FL-1101; Vector Laboratories, Inc) to detect capillaries, anti-SMA-Cy3 antibody (#C6198; Sigma-Aldrich) to detect arterioles, and wheat germ agglutinin (WGA)-CF350 (#29021-1; Biotium) to detect myofibers. Capillaries were defined as GSL+ vessels with a diameter <5 μm. Both capillary to fiber ratio (C:F) and capillary density (CD) were quantified to assess angiogenesis at the 14-day time-point. C:F was measured by counting the number of GSL+ capillaries relative to the number of WGA+ myofibers in a given field of view. CD was quantified by counting GSL+ capillaries per mm^2 of muscle. Lastly, microvascular area was measured using ImageJ software where the area of GSL+ vessels (detected by colour thresholding to generate a binary image) was measured and normalized to muscle cross-sectional area.

To assess arteriole number, the number of SMA+ vessels (defined as >5 μm diameter) was counted per mm^2 of muscle. To assess arteriole remodeling, SMA+ vessel diameter was measured using ImageJ software. Briefly, the tracing tool was used to delineate the perimeter of SMA+ arterioles and the subsequent conversion into diameter (μm). Capillary-sized SMA+ vessels (defined as <5 μm diameter) was also measured by counting co-stained GSL+ and SMA+ vessels per mm^2 of muscle.

2.6.3 Myofiber Assessment

To assess myofiber cross-sectional area, WGA staining was used to detect the cell membrane and outer boundaries of myocytes in 14-day post-ligation EDL. Briefly, ImageJ software was used to trace the perimeter of approximately 90% of all myofibers in the muscle cross-sectional fields of view and measurements were converted into area (μm^2).

2.6.4 Inflammation

To assess differences in immune cell infiltration in the 4-day post-ligated muscle, Hematoxylin and Eosin (H&E) staining of EDL cross sections was conducted where nuclei and myocyte cytoplasm was detected by hematoxylin and eosin, respectively (Mayer's Haematoxylin: #26306-01; Eosin Y: #26051-10, Electron Microscopy Sciences). Images were captured using an Olympus microscope (4X objective) and colour CCD camera. Immune cell infiltration area was measured by tracing clusters of nuclei found within the extracellular matrix. Infiltration area was then expressed relative to muscle cross sectional area (mm^2).

2.6.5 Ectopic Skeletal Muscle Fibrosis and Adipogenesis

To detect skeletal muscle fibrosis, EDL muscle subjected to 14 days of ischemia was stained using Masson's Trichrome following the manufacturer's protocol (Polysciences, Inc. 25088-1). Briefly, EDL cryosections were fixed with 10% formalin and subsequently washed with Wiegerts Iron Hematoxylin solution to detect nuclei (stained in black). Subsequently, sections were stained with Biebrich Scarlet-Acid Fuchsin solution to detect myofiber cytoplasm (stained in red). Lastly, sections were washed with Aniline Blue solution to detect fibrillar collagen within the interstitial space of the muscle (stained in blue). Images were captured using an Olympus microscope (4X objective) and colour CCD camera. Using ImageJ software, the area of collagen (detected by blue colour thresholding to generate a binary image) was measured and normalized to the area of the total muscle cross-section. The quantification of collagen deposition was expressed as a ratio of collagen area to muscle cross-sectional area. Ectopic adipocyte deposition was then measured within the interstitial spaces of the muscle. Regions of the section devoid of myofibers and blue-enriched fibrotic regions were defined as fibro-adipo deposits (white areas). Through colour

thresholding (ImageJ), the area of fibro-adipo regions was measured as a percentage of the muscle cross-sectional area.

2.7 RNA Analyses

Gastrocnemius muscle of EC-FoxO1 KD, EC-FoxO1,3 KD and control mice subjected to 4 and 14 days of ischemia was collected and lysed for RNA extraction using Qiagen RNeasy Mini Kit (#74106; Qiagen Inc). RNA concentrations and purity was then quantified. Reverse Transcription (RT) reaction was then used to synthesize cDNA (from 120 ng of mRNA) using M-MLV reverse transcriptase (#M0253, New England Biolabs). cDNA was further processed by real-time polymerase chain reaction (qPCR) which included Taqman probes and qPCR mastermix (#4444963; ThermoFisher Scientific) on a Qiagen Roto-Gene Q platform. All RNA probes were normalized to TATA-box binding protein (*Tbp;* Mm00446973_m1) mRNA due to its relatively consistent expression profile within the ischemic muscle. Transcript levels were calculated as $2^{-\Delta Ct}$.

2.7.1 Inflammatory Markers

RNA collected from gastrocnemius muscle subjected to 4 days of ischemia was assessed for markers of M1- and M2-like macrophages. M1 probes included *Cd68* (Mm00839636_g1) and integrin alpha x (*Itgax*; Mm00498701_m1). M2 probes used were transforming growth factor beta 1 (*Tgfb1;* Mm01178820_m1) and mannose receptor 1 (*Mrc1;* Mm00485148_m1). Lastly, endothelial cell Vascular Cell Adhesion Molecule (*Vcam1;* Mm01320970_m1) was used.

2.8 Statistics

Results were expressed as mean ± SEM. Blood flow recovery, H&E and immunofluorescence histology, as well as mRNA was analyzed using a two-way ANOVA repeated measures followed

by Bonferroni post hoc tests (Prism4; Graphpad Software Inc.) Post-ligation blood flow, and fibrotic/adipogenic area was analyzed using a two-tailed Student's *t* test. A $P<0.05$ was considered to represent a statistical significance.

Chapter 3: Results

3.1 Severe Hind Limb Ischemia Enhances Skeletal Muscle Damage and Inflammation

Skeletal muscle morphology and immune cell infiltration in the early stages of ischemia was assessed using H&E staining of EDL muscle. In non-ischemic EDL, myocytes contain peripheral nuclei (black), an enriched cytoplasmic protein staining with eosin (pink) and myofibers are arranged in highly ordered bundles surrounded by an intact extracellular matrix (white) (Figure 3.1A). These qualities are indicative of a healthy skeletal muscle. In 4-day ischemic EDL muscle, extensive muscle damage was observed. Myofibers were denucleated, appeared to be smaller in size and necrotic with pale eosin staining. Additionally, inflammatory cell nuclei were present and myofibers lost their ordered bundles, potentially due to ECM degradation and/or myofiber membrane lysis (Paoni et al., 2002; Toumi et al., 2006b). Lastly, adipocyte accumulation within the ECM was detected in regions enriched with inflammatory cell nuclei (Figure 3.1B).

Immune cell signatures, based on H&E and mRNA expression of macrophage markers *Itgax* and *Mrc1,* were not detected in non-ischemic muscle. However, immune cell infiltration area was significantly higher in ischemic compared to non-ischemic muscle, irrespective of mouse genotype (Figure 3.1C). To establish a more comprehensive understanding of macrophage phenotypes, gene expression of M1- and M2-macrophage markers were assessed in 4-day post-ligation gastrocnemius muscle. M1-macrophage marker *Itgax* expression was enhanced approximately 20-fold in the ischemic muscle, irrespective of genotype (Figure 3.1D). Following the same trend, M2-macrophage marker *Mrc1* was significantly elevated (15-fold) in the ischemic muscle in all genotypes (Figure 3.1E). Interestingly, M2-like growth factor *Tgfb1* was significantly upregulated in the ischemic muscle of EC-FoxO1,3 KD and EC-FoxO1 KD mice but not in Controls (Figure

3.1F). Altogether, this suggests the absence of EC-FoxO proteins may not alter the presence of M1 or M2 macrophages in ischemic muscle but can increase the enrichment of M2-like growth factor TGFβ1.

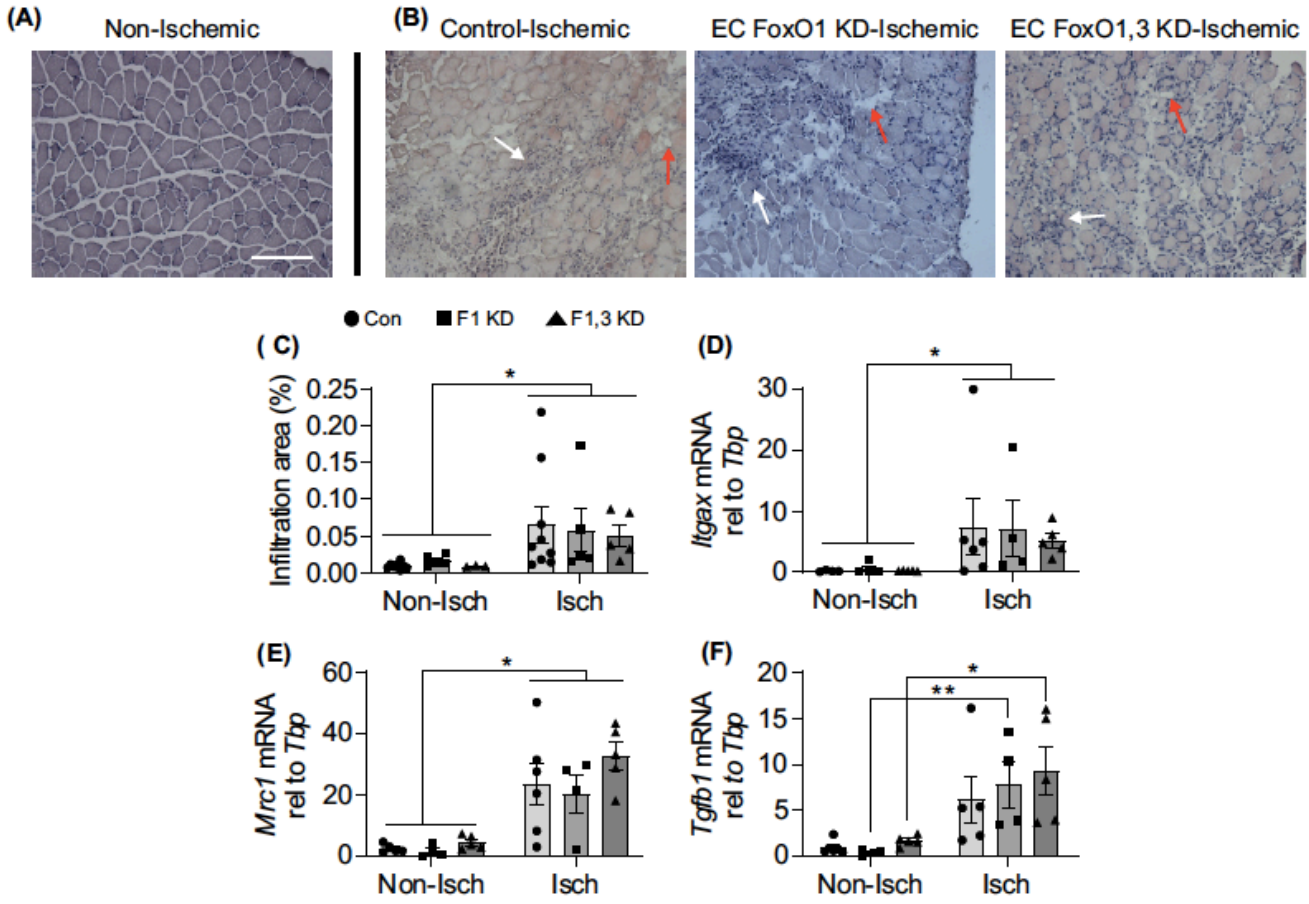

Figure 3.1: Skeletal muscle inflammation at 4 days post-ligation. H&E staining of non-ischemic (A) and ischemic (B) EDL muscle was used to assess skeletal muscle morphology and immune cell infiltration area (white arrows). Regions that may represent adipocytes are demarcated with red arrows. Immune cell infiltration was quantified as a percentage of the cross-sectional area of the muscle (C). mRNA levels of M1-macrophage marker *Itgax* (D) M2-macrophage marker *Mrc1* (E) and M2-like growth factor *Tgfb1* (F) were analyzed in non-ischemic (Non-isch) and ischemic (Isch) gastrocnemius muscle. **P*<0.05, ***P*<0.05 vs respective non-ischemic limb, two-way ANOVA with Bonferroni post-hoc test. EDL, extensor digitorum longus; H&E, haematoxylin and eosin; Con, Control; F1 KD, EC-FoxO1 KD; F1,3 KD, EC-FoxO1,3 KD.

3.2 Mice with Combined Endothelial FoxO1 and FoxO3 Deficiency Experience Enhanced Blood Flow Recovery and Microvascular Remodelling

Blood flow recovery up to 14 days post-ligation was assessed using Laser Doppler perfusion imaging (Figure 3.2A,C). Femoral artery ligation significantly reduced blood flow to the paw and leg (10% of contralateral limb) (Figure 3.2B,D). In all mice, paw blood flow improved from 0 to 4 days post-ligation and recovery plateaued beyond this time-point. However, compared to Control and EC-FoxO1 KD mice, paw perfusion was significantly higher in the ischemic hindlimb of EC-FoxO1,3 KD mice at days 4 and 7 post-ligation (Figure 3.2B). Since the paw lacks larger muscle groups (low metabolic activity) and is predominantly composed of skin and connective tissue (Wong et al., 2006), leg perfusion recovery was also assessed to more accurately represent bulk blood flow to the muscles. All mice improved leg blood flow at days 4 and 7 but no significant recovery beyond this time-point was detected in Control and EC-FoxO1 KD mice. Interestingly, EC-FoxO1,3 KD mice experienced greater perfusion recovery of the leg compared to Control and EC-FoxO1 KD mice at days 4, 7 and 14 of ischemia (Figure 3.2D).

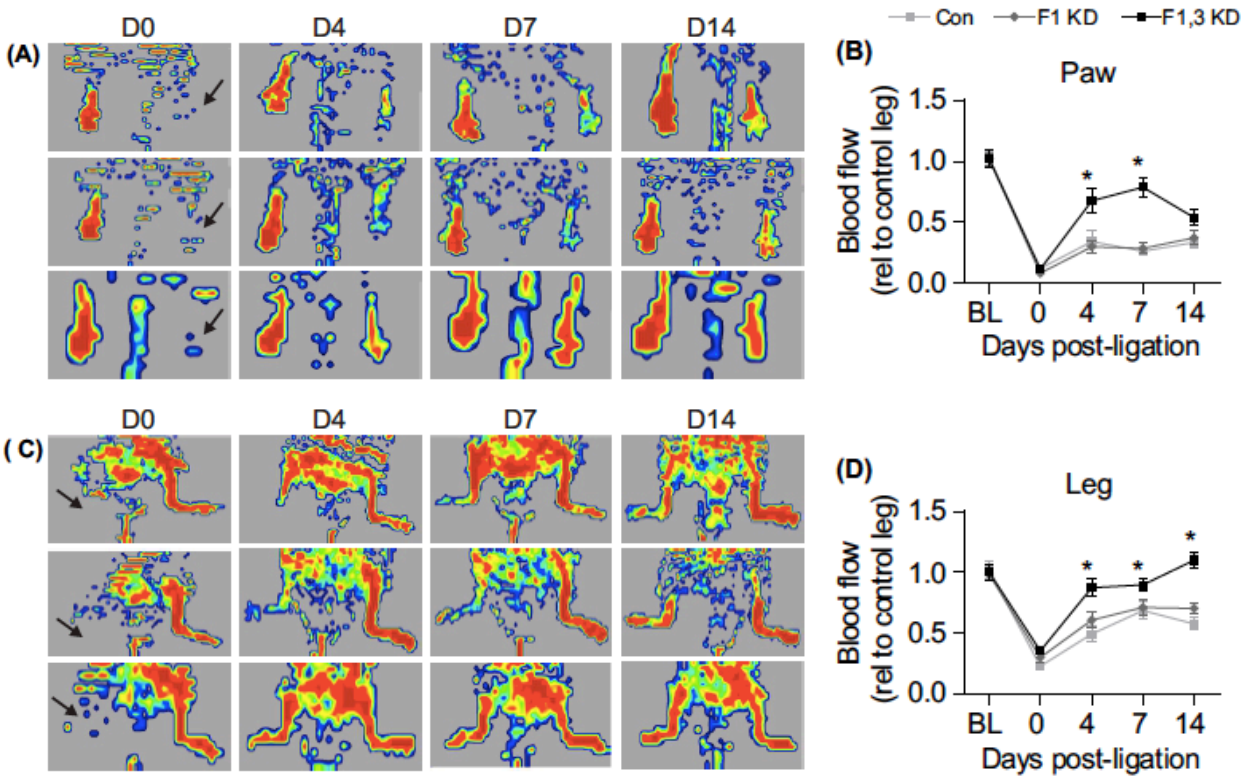

Figure 3.2: Blood flow recovery in ischemic limbs at 14 days post-ligation. Representative laser Doppler images of the dorsal paw (A) and ventral leg (C) regions immediately following ligation (day 0) and at days 4, 7 and 14 post-ligation. Black arrows indicate the ligated limbs. Quantification of perfusion was represented as a ratio between ischemic and non-ischemic limbs in the paw (B) and leg (D). **P*<0.05, vs Control and EC-FoxO1-KD mice, two-way ANOVA with Bonferroni post-hoc test. Con, Control; F1 KD, EC-FoxO1 KD; F1,3 KD, EC-FoxO1,3 KD.

Histological assessments were further used to investigate microvascular remodeling in ischemic EDL muscle. Representative images of capillaries in non-ischemic and ischemic EDL muscle 14 days post-ligation is shown (Figure 3.2A). At this time-point, both capillary-to-fiber ratio and capillary density (#/mm^2) were significantly elevated in ischemic muscle of EC-FoxO1,3 KD mice only (*P*<0.05; Figure 3.2B,C). However, we considered that high numbers of transverse capillaries may underestimate capillary content when expressed as capillary-to-fiber ratio or capillary density. Therefore, we also calculated microvascular area relative to muscle cross-sectional area. Compared to non-ischemic muscle, microvascular area was elevated in the ischemic muscle of all

mouse cohorts (P<0.05), but EC-FoxO1,3 KD mice exhibited significantly higher microvascular areas compared to their genotype counterparts (P<0.05; Figure 3.2D). These results suggest the combined action of EC-FoxO1 and EC-FoxO3 are more crucial in restraining post-ischemic muscle angiogenesis as opposed to EC-FoxO1 alone.

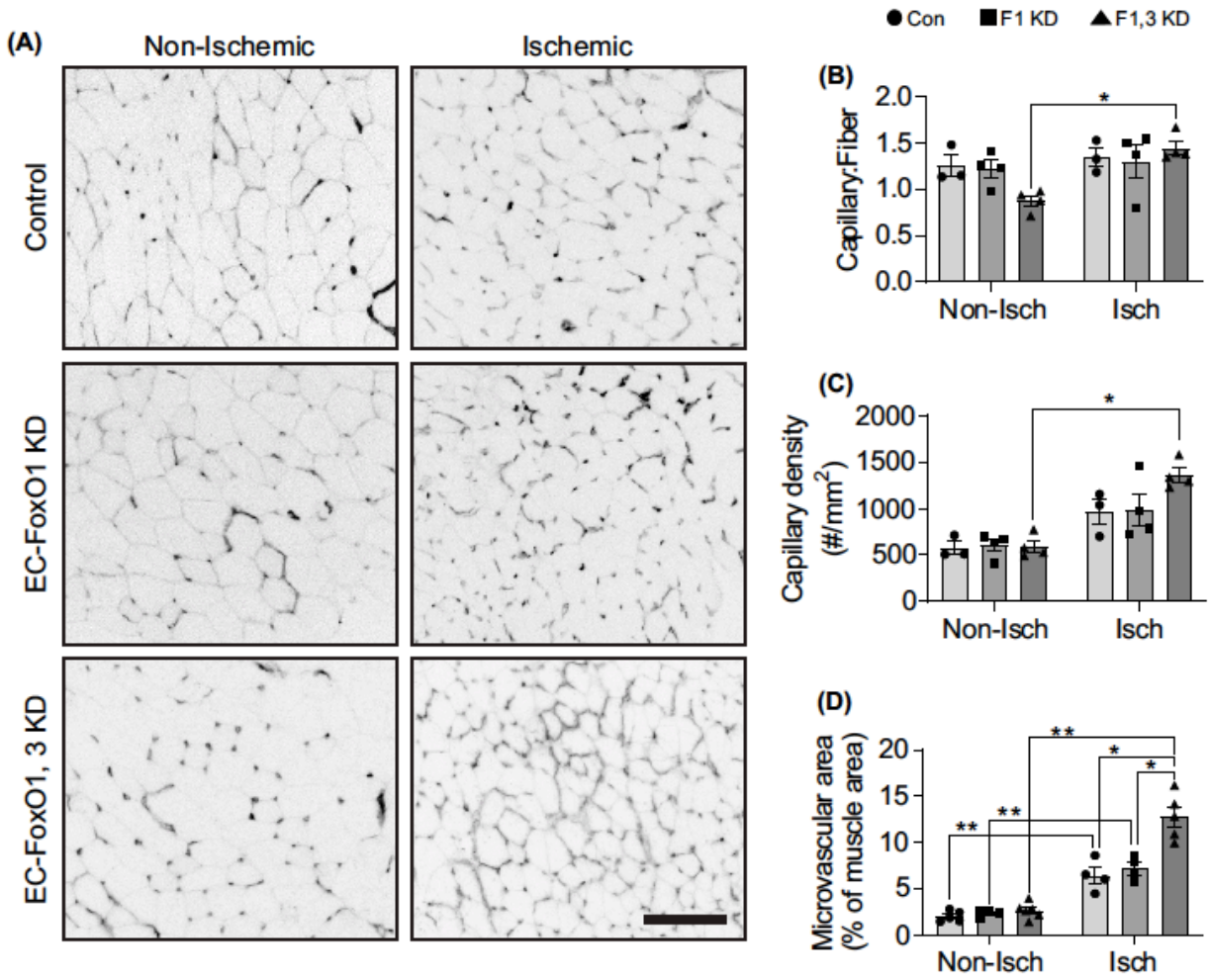

Figure 3.3: Enhanced capillary growth in ischemic muscle of EC-FoxO1,3-KD mice at 14 days post-ligation. Representative images of *Griffonia simplicifolia* lectin I (GSL)-FITC staining (to detect capillaries) in non-ischemic (Non-Isch) and ischemic (Isch) EDL muscle of Control, EC-FoxO1-KD and EC-FoxO1,3 KD mice (A). Capillary-to-fiber ratio (B), capillary density (C) and microvascular area (D) were measured in one to two fields of view (10x objective) per muscle. Scale bar = 100 µm. **P<0.05 vs respective non-ischemic limb, *P<0.05 vs comparative genotype ischemic limb, two-way ANOVA with Bonferroni post-hoc test. EDL, extensor digitorum longus. Con, Control; F1 KD, EC-FoxO1 KD; F1,3 KD, EC-FoxO1,3 KD.

Smooth muscle actin (SMA) staining of 14-day post-ligated EDL muscle is shown in representative images (Figure 3.4A). This was used to detect small capillary-sized SMA+ vessels which may represent neo-arterioles (diameter < 5 µm) (Peirce and Skalak, 2003) and larger SMA+ arterioles (diameter > 5 µm). In all mice cohorts, the SMA+ capillary density (#/mm^2 of muscle) was significantly higher in ischemic compared to non-ischemic muscle (Figure 3.4B). Interestingly, the density of larger arterioles (#/mm^2 of muscle) was significantly higher in the ischemic compared to non-ischemic muscle of EC-FoxO1,3 KD mice only (P<0.05; Figure 3.4C). Lastly, the average diameter of larger arterioles did not change with ischemia or genotype (Figure 3.4D). Overall, this suggests the combined absence of EC-FoxO1 and EC-FoxO3 can enhance arteriole numbers in post-ischemic regenerating muscle.

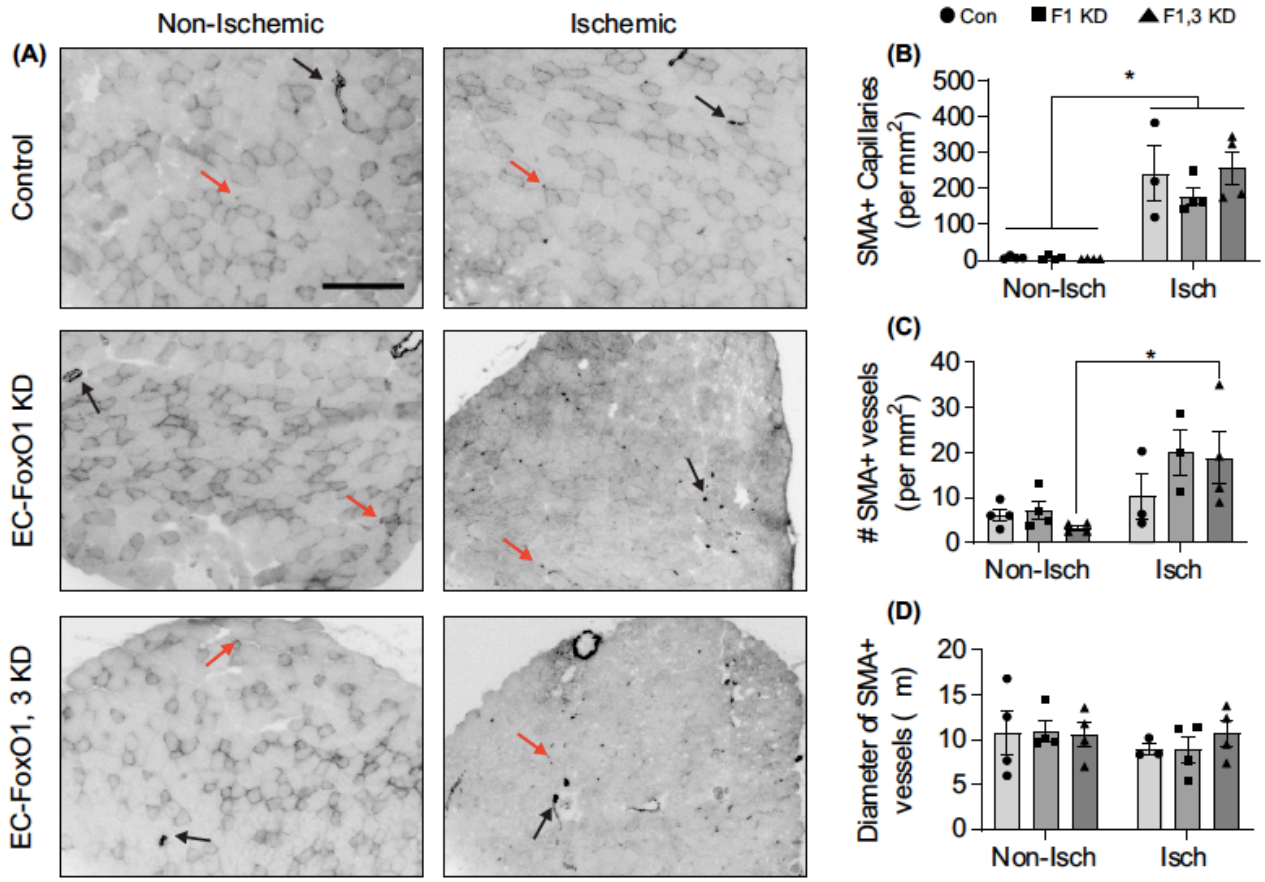

Figure 3.4: Greater number of arterioles in EC-FoxO1,3-KD muscle at 14 days post-ligation. Representative images of Cy3-anti-αSMA staining (to detect arterioles) in non-ischemic (Non-Isch) and ischemic (Isch) EDL muscle of Control, EC-FoxO1-KD and EC-FoxO1,3 KD mice (A). SMA+ capillary (<5 μm in diameter, red arrows) density (B), larger arteriole (>5 μm in diameter, black arrows) density (C) and arteriole diameter (D) were quantified in one to two fields of view (10x objective). Scale bar = 100 μm. **P*<0.05 vs respective non-ischemic limb, two-way ANOVA with Bonferroni post-hoc test. EDL, extensor digitorum longus. Con, Control; F1 KD, EC-FoxO1 KD; F1,3 KD, EC-FoxO1,3 KD.

3.3 Post-Ischemic Myofiber Maturation is Enhanced in EC-FoxO1,3 KD Mice

To assess skeletal muscle maturation, myofiber cross-sectional area was measured in 14-day post-ligation EDL muscle using WGA staining (Figure 3.5A). Following ischemia, there was a significant reduction in myofiber cross-sectional area (μm^2) in all three genotypes which is indicative of skeletal muscle atrophy and early stages of recovery (Figure 3.5B). However, Control and EC-FoxO1 KD mice had higher baseline myofiber cross-sectional areas compared to EC-FoxO1,3 KD mice, signifying an inherent difference in skeletal muscle morphology prior to ischemia. Therefore, to account for this and to more accurately assess the extent of skeletal muscle maturation in each mouse cohort, the fold change (ischemic/non-ischemic muscle) of myofiber cross-sectional area was quantified. Interestingly, EC-FoxO1,3 KD mice trended towards a higher ratio (~0.58) compared to Control (~0.3) mice (P=0.056) and was significantly higher compared to EC-FoxO1 KD (~0.3) mice (*P*<0.05; Figure 3.5C). This suggests that EC-FoxO1,3 KD ischemic muscle improved to baseline myofiber cross-sectional areas faster than their genotype counterparts which could be indicative of greater post-ischemic muscle maturation.

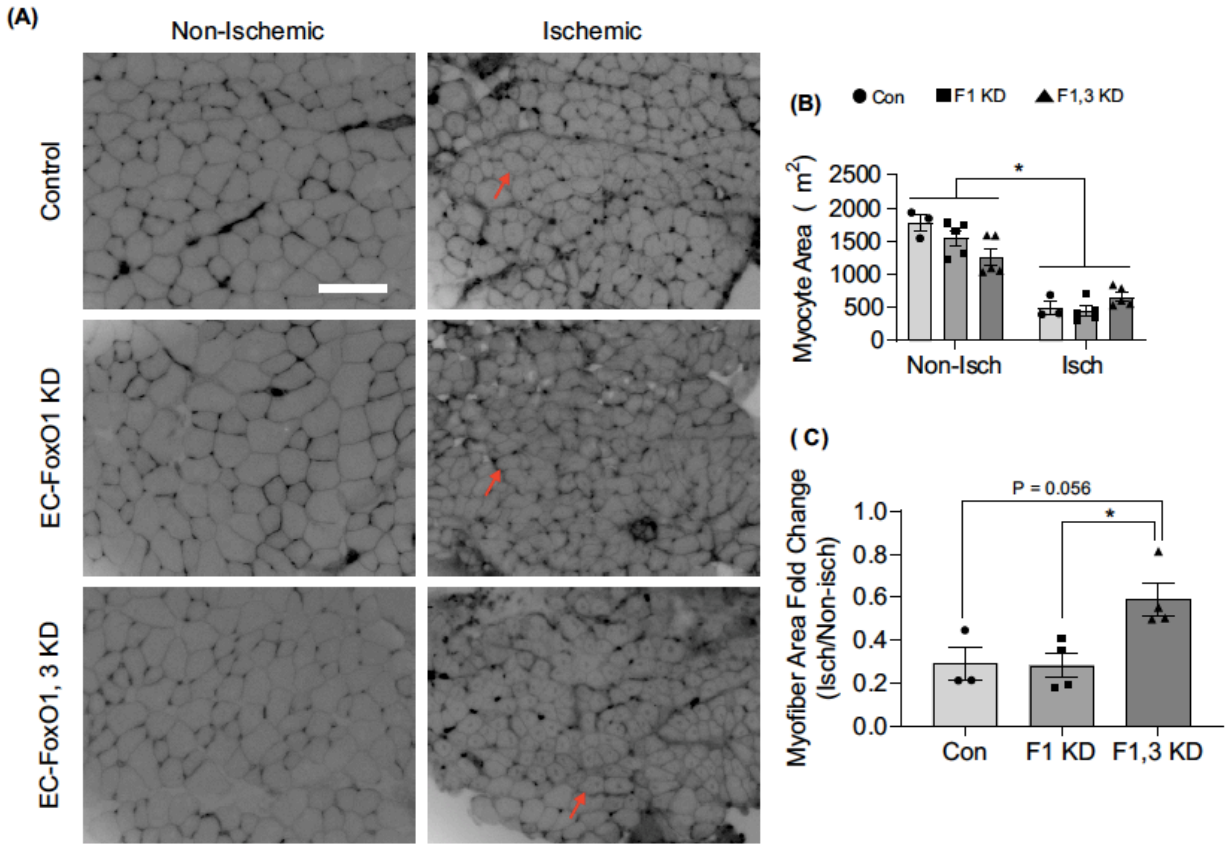

Figure 3.5: Myofiber cross-sectional area is greater in EC-FoxO1,3 KD muscle at 14 days post-ligation. Representative images of WGA-CF350 staining (to detect myofiber membranes) in non-ischemic (Non-Isch) and ischemic (Isch) EDL muscle of Control, EC-FoxO1-KD and EC-FoxO1,3 KD mice (A). Myofiber cross-sectional areas were assessed in one to two fields of view taken using a 10x objective (B). Myofiber area fold change (C), quantified as a ratio between ischemic and non-ischemic myofiber areas in each mouse. Scale bar = 100 µm. *P<0.05 vs respective non-ischemic limb, two-way ANOVA with Bonferroni post-hoc test. EDL, extensor digitorum longus. Con, Control; F1 KD, EC-FoxO1 KD; F1,3 KD, EC-FoxO1,3 KD.

3.4 Endothelial FoxO1 and 3 Synergistically Enhance Collagen Deposition in the Ischemic Muscle

Fibro-adipogenic and fibrotic remodeling was assessed in regenerating ischemic muscle using Massons trichrome staining of 14-day post-ligation EDL muscle (Figure 3.6A). Fibro-adipocyte accumulation was detected in ischemic muscle and quantified as a percentage of muscle area, but no significant differences were observed between mice genotypes (Figure 3.6B). Interestingly, however, fibrotic area was significantly greater in EC-FoxO1,3 KD ischemic muscle compared to Control and EC-FoxO1 KD ischemic muscle (P<0.05; Figure 6C).

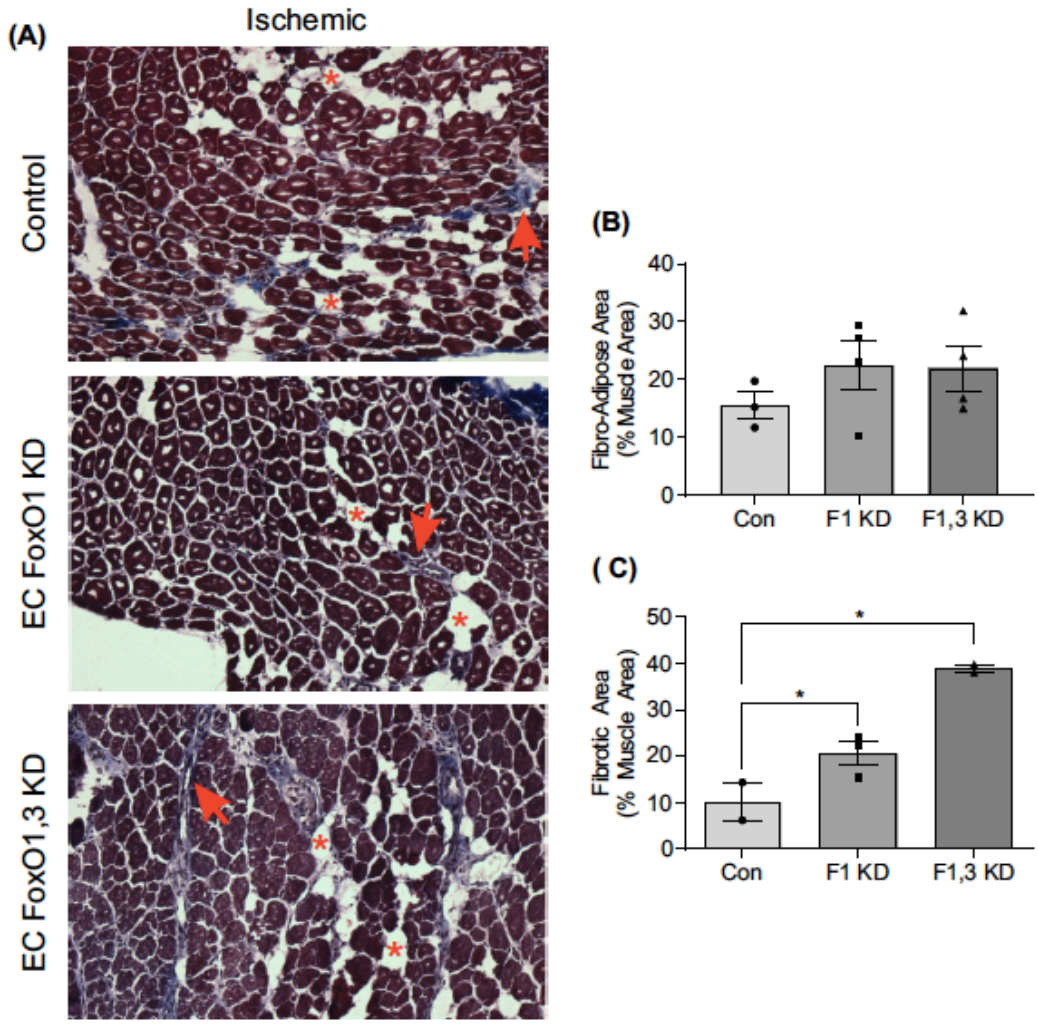

Figure 3.6: Enhanced fibrosis in EC-FoxO1,3 KD muscle at 14 days post-ligation. Masson's trichrome staining was used to detect fibrillar collagen (representing fibrosis) and fibro-adipogenesis (A). Fibro-adipocyte (B) and fibrosis (C) accumulation was quantified as an area (%) relative to muscle cross-sectional area in ischemic EDL of Control, EC-FoxO1-KD and EC-FoxO1,3 KD mice. Asterisk indicate fibro-adipogenic regions devoid of blue staining whereas red arrows demarcate areas of fibrosis (stained in blue). **P*<0.05, ***P*<0.05 vs Control and EC-FoxO1-KD, respectively. One-way ANOVA with Bonferroni post-hoc test. EDL, extensor digitorum longus. Con, Control; F1 KD, EC-FoxO1 KD; F1,3 KD, EC-FoxO1,3 KD.

Chapter 4: Discussion

4.1 Interpretation of Results

In this study, we establish that male EC-FoxO1,3 KD mice showed multiple signs of improvements at day 14 of post-ischemic muscle recovery compared to Control and EC-FoxO1 KD mice. By using histological and functional analyses, we reveal that EC-FoxO1,3 KD mice have enhanced (a) microvascular growth; (b) blood flow recovery of the paw and leg; and (c) a relative increase in myofiber cross-sectional area in the late stages of muscle ischemia. However, we also observed a potential deleterious outcome as EC-FoxO1,3 KD mice had more interstitial fibrosis in regenerating muscle compared to their genotype counterparts. These findings suggest that altering the biological profile of ECs through EC-FoxO-depletion can have profound influences in ischemic muscle outcomes.

EC-FoxO1,3 KD mice had elevated number of capillaries (indicator of angiogenesis) within the ischemic muscle compared to Control and EC-FoxO1 KD mice. By directly comparing these genotypes, we provide evidence that the combined depletion of EC-FoxO1 and 3 improved post-ischemic angiogenesis to a greater extent than EC-FoxO1 depletion alone. These findings are in line with our original hypothesis. Multiple explanations for this outcome can be speculated. For one, blood flow can serve as a stimulus for capillary growth through EC shear stress signalling (Egginton et al., 2016). Within the context of our current study, enhanced blood flow recovery in the early stages of ischemia (day 4 and 7) in EC-FoxO1,3 KD mice might have triggered shear stress-induced microvascular remodelling observed at day 14. Secondly, the single depletion of EC-FoxO1 might not have been sufficient to supress the angiostatic functions of EC-FoxO proteins. For example, EC-FoxO3 might have continued to promote the expression of angiostatic

genes, related to cell-cycle arrest and apoptosis, in the absence of EC-FoxO1 (Eijkelenboom and Burgering, 2013).

It was exciting to observe the enhancement of post-ischemic paw and leg perfusion recovery in EC-FoxO1,3 KD mice compared to Controls and EC-FoxO1 KD mice. It is well established that blood flow recovery can be improved with more arterioles within the ischemic muscle. This is because higher number of arterioles can reduce skeletal muscle microvascular resistance and facilitate greater flow from upstream vessels. As such, enhanced perfusion in EC-FoxO1,3 KD mice may partly be explained by the increased number of arterioles (large SMA+ vessels) within the ischemic muscle. Reports show that severe hind limb ischemia blunts vasodilation of the microvasculature and can reduce muscle perfusion (Arpino et al., 2017). Although not assessed in our study, it is possible that vasodilation was higher in EC-FoxO1,3 KD mice. It is possible that the absence of EC-FoxO1 and 3 (negative regulators of eNOS) is increasing the bioavailability of NO which can mediate NO-dependant arteriole vasodilation (Potente et al., 2005). Nevertheless, further investigation to elucidate if EC-FoxO1 and 3 depletion can modulate post-ischemic vasodilation and collateral artery remodelling would be required.

EC-FoxO1,3 KD mice had higher numbers of SMA+ vessels (> 5 μm) in the ischemic muscle, suggesting enhanced arteriolargenesis relative to their genotype counterparts. Numerous studies report that an M2-biased microenvironment can initiate a pro-arteriogenic program (Krishnasamy et al., 2017). We previously demonstrated that high-fat fed mice with an endothelial-specific combined depletion of FoxO1, 3 and 4 showed an M2-biased expression signature within the plantaris muscle (Nwadozi et al., 2020). As such, we anticipated that a similar shift would be

detectable in the microenvironment of EC-FoxO1,3 KD ischemic muscle. However, our current data suggest no differences in the M1/M2 profile, as *Itgax* and *Mrc1* expression levels were similar amongst all mouse cohorts. Another potential mechanism for improved arteriolargenesis can be related to the presence of TGFβ1, which is known to stimulate VSMC proliferation as well as differentiate pericytes into VSMCs, both of which contribute to arteriole arteriolargenesis (Chambers et al., 2003; Tsai et al., 2009). Our present study shows TGFβ1 levels were elevated in day 4 ischemic muscle in both EC-FoxO1 KD and EC-FoxO1,3 KD mice, therefore, this cannot explain the difference in arteriole growth between these cohorts. This also suggests that other pro-arteriogenic growth factors or cytokines may be responsible for the higher arteriolargenesis observed in EC-FoxO1,3 KD mice. Altogether, a more comprehensive assessment of the M1/M2 expression profile in 4- and 14-day ischemic muscle will be necessary to further elucidate the role of EC-FoxO proteins in mediating cytokine/growth factor-dependent arteriolargenesis.

We observed that ischemic muscle of EC-FoxO1,3 KD mice approached non-ischemic myofiber cross-sectional areas to a greater extent than their genotype counterparts. This trend may be indicative of either enhanced skeletal muscle maturation or less post-ischemic myofiber atrophy. Nevertheless, it can be postulated that this phenotype is a result of the concomitant increases in blood perfusion, angiogenesis and arteriolargenesis in EC-FoxO1,3 KD mice, which are known to be crucial for myofiber growth and overall health in trauma and hind limb ischemia models (Fernando et al., 2019; Hourdé et al., 2006). For example, enhancing arteriolargenesis in mice subjected to severe hind limb ischemia not only improved blood flow but also resulted in accelerated myofiber regeneration (Krishnasamy et al., 2017). It was also reported that microvascular perfusion recovery within patent capillary networks (detected with intravital

microscopy) closely paralleled skeletal muscle maturation following injury (Fernando et al., 2019). Enhanced blood flow and the distribution of this flow to individual myofibers (via capillaries) ensures the regenerating myofibers receive adequate oxygen, amino acids and growth factors (e.g. insulin) that are known to stimulate the myogenic program (Timmerman et al., 2010). For example, severe hypoxia impairs MuSC proliferation and myoblast differentiation (Chaillou and Lanner, 2016). This phenomenon can be extended to the context of hind limb ischemia, where more blood flow would minimize tissue-level hypoxia and facilitate myogenesis (Csete et al., 2001; Di Carlo et al., 2004). Taken together, the enhanced post-ischemic blood perfusion in EC-FoxO1,3 KD mice can likely improve myofiber growth through these mechanisms but further work is necessary to test this hypothesis.

The observed increase in post-ischemic muscle fibrosis in EC-FoxO1 KD and EC-FoxO1,3 KD mice can be explained potentially by an enrichment in a pro-fibrotic microenvironment. Based on our observations, enhanced TGFβ1 levels in EC-FoxO1KD and EC-FoxO1,3 KD mice might have been sufficient to promote fibrosis within the ischemic muscle. For one, TGFβ1 has been shown to stimulate fibroblasts and M2-macrophages to produce and secrete collagen within the ECM of regenerating muscle (Petrov et al., 2002; Tidball, 2017). Secondly, TGFβ1 derived from the ischemic microenvironment and/or endothelium of EC-FoxO1,3 KD mice can stimulate vascular smooth muscle cells (VSMCs) to proliferate. Interestingly, it was demonstrated that TGFβ1-stimulated VSMC proliferation results in a concomitant increase in VSMC-derived collagen production and deposition within the surrounding ECM due to VSMC transdifferentiation into myofibroblasts (Ismaeel et al., 2019). This is potentially why we observed more fibrosis surrounding the outer layer of larger arterioles in the masson's trichrome staining of EDL cross-

sections in EC-FoxO-depleted mice. We also observed that EC-FoxO1,3 KD mice had the highest fibrotic areas compared to their genotype counterparts. This suggests that the combined absence of EC-FoxO1 and 3 might promote other pro-fibrotic mechanisms in the ischemic muscle. We also measured potential fibro-adipogenic regions in 14-day ischemic EDL muscle which can be indicative of FAP cell differentiation as a result of persistent pro-inflammation (Lemos et al., 2015). We observed no differences between mouse cohorts which may suggest that FAP cells differentiated and committed to a more pro-fibrotic rather than a pro-adipogenic fate in ischemic muscle of EC-FoxO1 KD and EC-FoxO1,3 KD mice. However, we still detected some level of fibro-adipo accumulation and the differentiation of FAP cells into adipocytes should not be ruled out. Additionally, persistent pro-inflammation might not be influenced by the presence or absence of EC-FoxO proteins. Altogether, more investigation is required to further establish the role of EC-FoxO proteins in post-ischemic muscle fibrosis and adipocyte accumulation.

4.2 Study Limitations

I also acknowledge that our study has various limitations. For one, histological and biochemical analyses at day 4 and 14 post-ligation were not assessed in the same mice. As such, comparing analyses between day 4 and 14 can be subjected to error if there are inherent differences between the 4- and 14-day mouse cohorts. To minimize variability, mouse age, background and sex were matched at both time-points.

Additionally, biochemical analyses were measured in gastrocnemius muscle whereas histological assessments were conducted using EDL muscle. This was due to the relatively small size of the EDL (limited material) and the difficulty in conducting cross-sectional histology assessments in gastrocnemius muscle (with angled pennate fibers and multiple heads). However, this can lead to

sources of variability related to how ischemia can differentially affect EDL and gastrocnemius; both of which have inherent heterogeneity. For example, gastrocnemius muscle has a higher percentage (~6%) of type-I fibers (highly oxidative and more mitochondrial content) compared to EDL muscle (~0.2%) in C57BL6/J mice (Augusto et al., 2003). This may have introduced a significant source of variability as we previously demonstrated that oxidative potential can alter ischemic muscle outcomes (Nwadozi et al., 2020). Other differences include skeletal muscle mass and vascular supply from collateral vessels. To control for these differences, histological and biochemical analyses were conducted in regenerating zones of the muscle. Additionally, the femoral artery was ligated in the most proximal region, blocking most collateral vessel blood flow. Nevertheless, these limitations can be overcome in future experiments by conducting biochemical and histological analyses using the relatively larger tibialis anterior muscle.

Another limitation is that FoxO1$^{f/f}$ and FoxO*1,3*$^{f/f}$ mice were from separate colonies and differed in their extent of back-crossing. This led to a source of variability in which FoxO1$^{f/f}$ mice had more of an FVB/n background whereas *FoxO1,3*$^{f/f}$ mice had a more equal mix of FVB/n and C57Bl6/J. This heterogeneity may contribute to why we observed different base-line (non-ischemic muscle) measurements of myofiber cross-sectional areas and capillary to fiber ratios between the two cohorts. However, this variability was controlled by assessing differences between non-ischemic and ischemic muscle within each mouse cohort.

It is also worth mentioning that our current adipocyte quantification was a crude and non-accurate assessment as it relied on morphological features that do not represent specific adipogenic markers.

As such, this quantification can be improved in future studies by using histological techniques that directly measure the presence of adipocytes or lipids such as Oil Red O staining.

Lastly, some of our analyses have a low sample size (underpowered experiments), which may lead to Type 2 statistical error. This means it is difficult to conclude no differences, in a given parameter, exist between mouse cohorts. Within our study, this includes underpowered analyses such as myofiber cross-sectional areas and fibro-adipogenic areas that did not differ when directly comparing these parameters between genotypes. To overcome this limitation, we recently added more 14-day post-ligation mice to our study and will complete these assessments in the future.

4.3 Future Directions

Our study reveals that EC-FoxO1,3 KD mice have enhanced macro-level blood flow recovery. To determine if depleting EC-FoxO1 and 3 can improve microvascular perfusion, intravital microscopy techniques can be used to measure real-time arteriole and capillary hemodynamics. Similar to our previous work, capillary RBC velocity, lineal density, supply rate, RBC oxygen saturation and oxygen delivery rate can be measured in the capillary bed of ischemic muscle (Nwadozi et al., 2020). These assessments would provide insight into whether or not the patent capillary network of the ischemic muscle is functional and able to adequately meet the oxygen demands of regenerating myofibers. With more microvascular structures (responsible for local flow distribution) in EC-FoxO1,3 KD ischemic muscle, it could be hypothesized that this correlates with improved hemodynamics and perfusion at the level of the myofibers.

Blood perfusion recovery can also be mediated by improved vasodilation of the arterioles. Therefore, it can be hypothesized that EC-FoxO1 and 3 depletion can improve post-ischemic

vasodilation. EC-mediated vasodilation is predominately controlled by NO derived from eNOS activity (Baum et al., 2004). Therefore, measuring the relative abundance of eNOS in Control, EC-FoxO1 KD and EC-FoxO1,3 KD microvascular fragments could signify their respective vasodilatory potential. Potente et al. demonstrated that both EC-FoxO1 and 3 independently supress eNOS transcription (Potente et al., 2005). Therefore, eNOS levels are potentially higher in microvascular fragments harvested from EC-FoxO1,3 KD compared to EC-FoxO1 KD ischemic muscle.

Although our present study revealed EC-FoxO1,3 KD mice have greater post-ischemic angiogenesis, the specific mechanisms that explain this phenotype still need further investigation. EC-FoxO depletion can induce more angiogenesis by promoting indirect and/or direct mechanisms that additively increase capillary expansion. Indirectly, higher blood flow (from either vasodilation or arteriole growth) in EC-FoxO1,3 KD mice can enhance EC shear-stress signalling which can serve as a pro-angiogenic stimulus. We and others have demonstrated that EC-VEGFR2 auto-phosphorylates in response to shear stress and triggers a downstream angiogenic program (Chen et al., 1999; Gee et al., 2010; Jin Zheng-Gen et al., 2003). Therefore, it would be interesting to measure differences in activated VEGFR2 (and associated downstream signals) in ECs isolated from ischemic muscle and how this correlates with the cohorts respective angiogenic phenotype. More directly, EC-FoxO1 and 3 depletion might also be promoting more pro-angiogenic signals within the EC compared to the single absence of EC-FoxO1. To test this hypothesis, various mechanisms related to FoxO1 and 3 control of EC cell-cycle progression can be investigated. This includes measuring genes redundantly expressed by EC-FoxO1 and 3 including p21 and p27 which are responsible for cell-cycle arrest (Eijkelenboom and Burgering, 2013; Zhang et al., 2011).

Perhaps the expression of these genes is higher in EC-FoxO1,3– compared to EC-FoxO1-silenced ECs. Additionally, knowing that increased metabolism is required for EC cell-cycle progression (and hence angiogenesis), future investigations can be aimed to identify how EC-FoxO1 and 3 depletion can enhance this coupled response. One study reported that EC-FoxO1 supresses the function of c-MYC, lowering EC glycolytic and oxidative metabolism, and concomitantly reducing vascular growth (Wilhelm et al., 2016). However, this potential has not been established for EC-FoxO3. Therefore, it would be interesting to determine if EC-FoxO3 plays an additive role in downregulating c-MYC. To elucidate this mechanism, the metabolic profile and expression of c-MYC in ECs from Control, EC-FoxO1 KD and EC-FoxO1,3 KD ischemic muscle can be measured and correlated with their respective angiogenic phenotype.

Mechanisms for how EC-FoxO1 and 3 depletion can increase post-ischemic arteriole numbers can also be explored. Post-ischemic arteriogenesis requires a certain level of EC oxidative stress in the form of ROS (Tojo et al., 2005). Interestingly, FoxO proteins are commonly thought to lower ROS levels by upregulating anti-oxidant proteins SOD2 and CAT (Klotz et al., 2015). With regards to our current study, the combined absence of EC-FoxO1 and 3 can possibly increase ischemia-induced EC ROS production and, in turn, a pro-arteriogenic phenotype. Therefore, it would be intriguing to measure redox homeostasis in microvascular fragments of ischemic muscle to identify if EC-FoxO depletion can alter this profile. Additionally, arteriole growth can be facilitated by pericyte differentiation into VSMCs through TGFβ1 signalling (Chambers et al., 2003; Tsai et al., 2009). In future studies, it would be novel to elucidate if FoxO1,3-silenced ECs can promote this mechanism of differentiation when cultured with pericytes.

As a means to determine how EC-FoxO proteins can contribute to post-ischemic muscle regeneration, our current study assessed myofiber cross-sectional areas at day 14. To make a more robust conclusion, this assessment should be supplemented with other biochemical and histological analyses. This includes developing a time-line of myofiber cross-sectional area growth and an expression profile of myogenic regulatory factors (e. g. Pax7, Myf5 and MyoD) in the early and late stages of ischemia. This also includes adding a third mouse cohort subjected to 21 days of ischemia and conducting limb functional assessments such as whole-muscle contractility (Bonetto et al., 2015). This would allow us to assess the end stages of muscle recovery and the overall regaining of limb function.

Lastly, it would be novel to investigate mechanisms by which ECs can communicate with various cell-types of the skeletal muscle micro niche and how EC-FoxO1 and 3 depletion can alter their respective biological profiles. However, this can be challenging to conduct *in vivo*. Therefore, a series of *in vitro* cell culture experiments or harvesting Control, EC-FoxO1 KD and EC-FoxO1,3 KD ECs can be performed. Potential mechanisms include:

Endothelial-Immune Cell Crosstalk: It would be novel to determine if EC-FoxO-suppression can differentiate macrophages towards a pro- or anti-inflammatory phenotype. This can be achieved by culturing ECs with monocytes and measuring an M1/M2 expression profile. Interestingly, ECs were shown to differentiate circulating monocytes into M2 macrophages via juxtacrine Notch-Dll1 signalling in ischemic muscle (Krishnasamy et al., 2017). Therefore, elucidating if EC-FoxO1 and 3 proteins can modulate Notch-Dll1-mediated M2 signalling would be an intriguing next step.

Endothelial-Satellite Cell Crosstalk: Possible mechanisms to elucidate how EC-FoxO proteins can influence EC-MuSC cross-talk could also be investigated. FoxO1 and 3 regulation of EC metabolism can potentially mediate EC-MuSC crosstalk. It is known EC-FoxO1 and 3 depletion enhances EC metabolism when subjected to a growth stimulus (Rudnicki et al., 2018; Salih and Brunet, 2008). This results in a concomitant increase in the production and release of lactate from the EC. Interestingly, lactate promotes the differentiation and maturation of myotubes (Ohno et al., 2019). Therefore, this may serve as a potential paracrine mechanism coupling the function of EC-FoxO proteins and the myogenic program.

Endothelial-Fibroblast/FAP Cell Crosstalk: Cytokines regulated by EC-FoxO1 and 3 (e.g. TGFβ1) can influence the fibrotic or adipogenic potential of fibroblasts and/or FAP cells (Tidball, 2017). Potente et al. demonstrated that HUVECs with FoxO3 siRNA increased pro-fibtrotic cytokine IL-8 but this did not occur in FoxO1-silenced HUVECs (Potente et al., 2005). IL-8 can stimulate fibroblasts to release collagen within the ECM (De Paepe and De Bleecker, 2013). Therefore, IL-8 and collagen levels can be measured while culturing FoxO1- and FoxO1,3-silenced ECs with fibroblasts/FAP cells.

4.4 Conclusion and Significance

My thesis project provides a unique perspective that altering the biological profile of ECs, through EC-FoxO1 and 3 depletion, can influence post-ischemic vascular, myogenic and tissue remodelling events. We are also the first group to directly compare the singular role of EC-FoxO1 and the combined role of EC-FoxO1 and 3 in modulating these key events that characterize muscle recovery following severe hind limb ischemia. Based on our results, we conclude that EC-FoxO1 and 3 depletion enhances post-ischemic angiogenesis and arteriolargenesis, blood flow recovery

and the potential for enhanced myofiber growth. These positive responses were also correlated with enhanced accumulation of fibrotic tissue: a potentially deleterious result. Interestingly, all these outcomes were greater in magnitude in EC-FoxO1,3 KD compared to EC-FoxO1 KD mice. This suggests that compared to the single depletion of EC-FoxO1, the combined depletion of EC-FoxO1 and 3 has a more profound role in influencing ischemic muscle outcomes. Altogether, our overarching analyses provide a foundation for novel future investigations which include elucidating specific mechanisms for how EC-FoxO proteins can modulate EC cross-talk between various cells of the skeletal muscle micro-niche. From a clinical perspective, this can provide insight into a potential therapeutic target in managing the vascular, inflammatory and myogenic events that characterize CLI. Ultimately, my thesis project contributes to a growing body of knowledge that aims to develop an effective therapy with the end goal of promoting a more favorable PAD prognosis.

Reference List

Adair, T.H., and Montani, J.-P. (2010). Overview of Angiogenesis (Morgan & Claypool Life Sciences).

Aird, W.C. (2008). Endothelium in health and disease. Pharmacol. Rep. 5.

Anderson, S.I., Shiner, R., Brown, M.D., and Hudlicka, O. (2006). ICAM-1 expression and leukocyte behavior in the microcirculation of chronically ischemic rat skeletal muscles. Microvasc. Res. *71*, 205–211.

Annecke, T., Fischer, J., Hartmann, H., Tschoep, J., Rehm, M., Conzen, P., Sommerhoff, C.P., and Becker, B.F. (2011). Shedding of the coronary endothelial glycocalyx: effects of hypoxia/reoxygenation vs ischaemia/reperfusion. Br. J. Anaesth. *107*, 679–686.

Annex, B.H. (2013). Therapeutic angiogenesis for critical limb ischaemia. Nat. Rev. Cardiol. *10*, 387–396.

Aref, Z., de Vries, M.R., and Quax, P.H.A. (2019). Variations in Surgical Procedures for Inducing Hind Limb Ischemia in Mice and the Impact of These Variations on Neovascularization Assessment. Int. J. Mol. Sci. *20*.

Arpino, J.-M., Nong, Z., Li, F., Yin, H., Ghonaim, N., Milkovich, S., Balint, B., O'Neil, C., Fraser, G.M., Goldman, D., et al. (2017). Four-Dimensional Microvascular Analysis Reveals That Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation. Circ. Res. *120*, 1453–1465.

Baum, O., Da Silva-Azevedo, L., Willerding, G., Wöckel, A., Planitzer, G., Gossrau, R., Pries, A.R., and Zakrzewicz, A. (2004). Endothelial NOS is main mediator for shear stress-dependent angiogenesis in skeletal muscle after prazosin administration. Am. J. Physiol.-Heart Circ. Physiol. *287*, H2300–H2308.

Ben-Shoshan, J., Maysel-Auslender, S., Luboshits, G., Barshack, I., Polak-Charcon, S., Tzahor, E., Keren, G., and George, J. (2009). Hypoxia-Inducible Factor-1α and -2α Additively Promote Endothelial Vasculogenic Properties. J. Vasc. Res. *46*, 299–310.

Bentzinger, C.F., Wang, Y.X., Dumont, N.A., and Rudnicki, M.A. (2013). Cellular dynamics in the muscle satellite cell niche. EMBO Rep. *14*, 1062–1072.

Bergmann, C.E., Hoefer, I.E., Meder, B., Roth, H., van Royen, N., Breit, S.M., Jost, M.M., Aharinejad, S., Hartmann, S., and Buschmann, I.R. (2006). Arteriogenesis depends on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. J. Leukoc. Biol. *80*, 59–65.

Biewener, A.A. (2016). Locomotion as an emergent property of muscle contractile dynamics. J. Exp. Biol. *219*, 285–294.

Blanco, R., and Gerhardt, H. (2013). VEGF and Notch in Tip and Stalk Cell Selection. Cold Spring Harb. Perspect. Med. *3*.

Bonetto, A., Andersson, D.C., and Waning, D.L. (2015). Assessment of muscle mass and strength in mice. BoneKEy Rep. *4*.

Brandão, M.L., Roselino, J.E.S., Piccinato, C.E., and Cherri, J. (2003). Mitochondrial alterations in skeletal muscle submitted to total ischemia. J. Surg. Res. *110*, 235–240.

Brenes, R.A., Jadlowiec, C.C., Bear, M., Hashim, P., Protack, C.D., Li, X., Lv, W., Collins, M.J., and Dardik, A. (2012). Toward A Mouse Model of Hind Limb Ischemia to Test Therapeutic Angiogenesis. J. Vasc. Surg. *56*, 1669–1679.

Brew, K., and Nagase, H. (2010). The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. Biochim. Biophys. Acta BBA - Mol. Cell Res. *1803*, 55–71.

Brigitte, M., Schilte, C., Plonquet, A., Baba-Amer, Y., Henri, A., Charlier, C., Tajbakhsh, S., Albert, M., Gherardi, R.K., and Chrétien, F. (2010). Muscle resident macrophages control the immune cell reaction in a mouse model of notexin-induced myoinjury. Arthritis Rheum. *62*, 268–279.

Bruce, A.C., Kelly-Goss, M.R., Heuslein, J.L., Meisner, J.K., Price, R.J., and Peirce, S.M. (2014). Monocytes are Recruited from Venules during Arteriogenesis in the Murine Spinotrapezius Ligation Model. Arterioscler. Thromb. Vasc. Biol. *34*, 2012–2022.

Burri, P.H., and Tarek, M.R. (1990). A novel mechanism of capillary growth in the rat pulmonary microcirculation. Anat. Rec. *228*, 35–45.

Carmeliet, P. (2003). Angiogenesis in health and disease. Nat. Med. *9*, 8.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., et al. (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat. Med. *7*, 575–583.

Carter, S.A., Hamel, E.R., Paterson, J.M., Snow, C.J., and Mymin, D. (1989). Walking ability and anlde systolic pressures" Observations in patients with intermittent claudication in a short-term walldng exercise program. *10*, 8.

Cebasek, V., KubÃ-novÃ¡, L., Ribaric, S., and Erzen, I. (2004). A novel staining method for quantification and 3D visualisation of capillaries and muscle fibres. Eur. J. Histochem. 151–158.

Chaillou1, T., and Lanner, J.T. (2016). Regulation of myogenesis and skeletal muscle regeneration: effects of oxygen levels on satellite cell activity. FASEB J. *30*, 3929–3941.

Chambers, R.C., Leoni, P., Kaminski, N., Laurent, G.J., and Heller, R.A. (2003). Global Expression Profiling of Fibroblast Responses to Transforming Growth Factor-β1 Reveals the Induction of Inhibitor of Differentiation-1 and Provides Evidence of Smooth Muscle Cell Phenotypic Switching. Am. J. Pathol. *162*, 533–546.

Chen, K.-D., Li, Y.-S., Kim, M., Li, S., Yuan, S., Chien, S., and Shyy, J.Y.-J. (1999). Mechanotransduction in Response to Shear Stress: ROLES OF RECEPTOR TYROSINE KINASES, INTEGRINS, AND Shc. J. Biol. Chem. *274*, 18393–18400.

Chiu, J.-J., and Chien, S. (2011). Effects of Disturbed Flow on Vascular Endothelium: Pathophysiological Basis and Clinical Perspectives. Physiol. Rev. *91*.

Christov, C., Chrétien, F., Abou-Khalil, R., Bassez, G., Vallet, G., Authier, F.-J., Bassaglia, Y., Shinin, V., Tajbakhsh, S., Chazaud, B., et al. (2007). Muscle Satellite Cells and Endothelial Cells: Close Neighbors and Privileged Partners. Mol. Biol. Cell *18*, 1397–1409.

Clarke, M.S., and Feeback, D.L. (1996). Mechanical load induces sarcoplasmic wounding and FGF release in differentiated human skeletal muscle cultures. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *10*, 502–509.

Claxton, S., Kostourou, V., Jadeja, S., Chambon, P., Hodivala-Dilke, K., and Fruttiger, M. (2008). Efficient, inducible Cre-recombinase activation in vascular endothelium. Genes. N. Y. N 2000 *46*, 74–80.

Cooke, J.P., and Dzau, V.J. (1997). Derangements of the nitric oxide synthase pathway, L-arginine, and cardiovascular diseases. Circulation *96*, 379–382.

Cornejo del Río, V., Mostaza, J., Lahoz, C., Sánchez-Arroyo, V., Sabín, C., López, S., Patrón, P., Fernández-García, P., Fernández-Puntero, B., Vicent, D., et al. (2017). Prevalence of peripheral artery disease (PAD) and factors associated: An epidemiological analysis from the population-based Screening PRE-diabetes and type 2 DIAbetes (SPREDIA-2) study. PLoS ONE *12*.

Couffinhal, T., Silver, M., Zheng, L.P., Kearney, M., Witzenbichler, B., and Isner, J.M. (1998). Mouse model of angiogenesis. Am. J. Pathol. *152*, 1667–1679.

Csete, M., Walikonis, J., Slawny, N., Wei, Y., Korsnes, S., Doyle, J.C., and Wold, B. (2001). Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. J. Cell. Physiol. *189*, 189–196.

Daitoku, H., Sakamaki, J., and Fukamizu, A. (2011). Regulation of FoxO transcription factors by acetylation and protein–protein interactions. Biochim. Biophys. Acta BBA - Mol. Cell Res. *1813*, 1954–1960.

Dang, C.V. (2013). MYC, Metabolism, Cell Growth, and Tumorigenesis. Cold Spring Harb. Perspect. Med. *3*.

De Paepe, B., and De Bleecker, J.L. (2013). Cytokines and Chemokines as Regulators of Skeletal Muscle Inflammation: Presenting the Case of Duchenne Muscular Dystrophy. Mediators Inflamm. *2013*.

De Spiegelaere, W., Casteleyn, C., Van den Broeck, W., Plendl, J., Bahramsoltani, M., Simoens, P., Djonov, V., and Cornillie, P. (2012). Intussusceptive Angiogenesis: A Biologically Relevant Form of Angiogenesis. J. Vasc. Res. *49*, 390–404.

Di Carlo, A., De Mori, R., Martelli, F., Pompilio, G., Capogrossi, M.C., and Germani, A. (2004). Hypoxia Inhibits Myogenic Differentiation through Accelerated MyoD Degradation. J. Biol. Chem. *279*, 16332–16338.

Dodge, J.T., Bevan, R.D., and Bevan, J.A. (1994). Comparison of density of sympathetic varicosities and their closeness to smooth muscle cells in rabbit middle cerebral and ear arteries and their branches. Circ. Res. *75*, 916–925.

Dragneva, G., Korpisalo, P., and Ylä-Herttuala, S. (2013). Promoting blood vessel growth in ischemic diseases: challenges in translating preclinical potential into clinical success. Dis. Model. Mech. *6*, 312–322.

Dumont, N.A., Wang, Y.X., and Rudnicki, M.A. (2015a). Intrinsic and extrinsic mechanisms regulating satellite cell function. Dev. Camb. Engl. *142*, 1572–1581.

Dumont, N.A., Bentzinger, C.F., Sincennes, M.-C., and Rudnicki, M.A. (2015b). Satellite Cells and Skeletal Muscle Regeneration. Compr. Physiol. *5*, 1027–1059.

Durand, M.J., and Gutterman, D.D. (2013). Diversity in Mechanisms of Endothelium-Dependent Vasodilation in Health and Disease. Microcirc. N. Y. N 1994 *20*, 239–247.

Egginton, S., Hussain, A., Hall-Jones, J., Chaudhry, B., Syeda, F., and Glen, K.E. (2016). Shear stress-induced angiogenesis in mouse muscle is independent of the vasodilator mechanism and quickly reversible. Acta Physiol. Oxf. Engl. *218*, 153–166.

Eijkelenboom, A., and Burgering, B.M.T. (2013). FOXOs: signalling integrators for homeostasis maintenance. Nat. Rev. Mol. Cell Biol. *14*, 83–97.

Fernando, C.A., Pangan, A.M., Cornelison, D., and Segal, S.S. (2019). Recovery of blood flow regulation in microvascular resistance networks during regeneration of mouse gluteus maximus muscle. J. Physiol. *597*, 1401–1417.

Frontera, W.R., and Ochala, J. (2015). Skeletal Muscle: A Brief Review of Structure and Function. Calcif. Tissue Int. *96*, 183–195.

Gao, Q., Chen, K., Gao, L., Zheng, Y., and Yang, Y.-G. (2016). Thrombospondin-1 signaling through CD47 inhibits cell cycle progression and induces senescence in endothelial cells. Cell Death Dis. *7*, e2368–e2368.

Gee, E., Milkiewicz, M., and Haas, T.L. (2010). p38 MAPK is Activated by Vascular endothelial growth factor receptor 2 and is essential for shear stress-induced angiogenesis. J. Cell. Physiol. *222*, 120–126.

Gerhardt, H. (2008). VEGF and endothelial guidance in angiogenic sprouting. Organogenesis *4*, 241–246.

Gordon, S., and Plüddemann, A. (2017). Tissue macrophages: heterogeneity and functions. BMC Biol. *15*.

Green, D.J., Hopman, M.T.E., Padilla, J., Laughlin, M.H., and Thijssen, D.H.J. (2017). Vascular Adaptation to Exercise in Humans: Role of Hemodynamic Stimuli. Physiol. Rev. *97*, 495–528.

Grimmer, M., Riener, R., Walsh, C.J., and Seyfarth, A. (2019). Mobility related physical and functional losses due to aging and disease - a motivation for lower limb exoskeletons. J. NeuroEngineering Rehabil. *16*.

Guo, K., Wang, J., Andrés, V., Smith, R.C., and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. Mol. Cell. Biol. *15*, 3823–3829.

Gustafsson, T., Knutsson, A., Puntschart, A., Kaijser, L., Nordqvist, A.-C.S., Sundberg, C.J., and Jansson, E. (2002). Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. Pflugers Arch. *444*, 752–759.

Gute, D., Fraga, C., Laughlin, M.H., and Amann, J.F. (1996). Regional changes in capillary supply in skeletal muscle of high-intensity endurance-trained rats. J. Appl. Physiol. *81*, 619–626.

Haas, T.L., and Nwadozi, E. (2015). Regulation of skeletal muscle capillary growth in exercise and disease. Appl. Physiol. Nutr. Metab. Physiol. Appl. Nutr. Metab. *40*, 1221–1232.

Haas, T.L., Milkiewicz, M., Davis, S.J., Zhou, A.L., Egginton, S., Brown, M.D., Madri, J.A., and Hudlicka, O. (2000). Matrix metalloproteinase activity is required for activity-induced angiogenesis in rat skeletal muscle. Am. J. Physiol. Heart Circ. Physiol. *279*, H1540-1547.

Hansen-Smith, F., Egginton, S., and Hudlicka, O. (1998). Growth of arterioles in chronically stimulated adult rat skeletal muscle. Microcirc. N. Y. N 1994 *5*, 49–59.

Hansen-Smith, F., Egginton, S., Zhou, A.-L., and Hudlicka, O. (2001). Growth of Arterioles Precedes That of Capillaries in Stretch-Induced Angiogenesis in Skeletal Muscle. Microvasc. Res. *62*, 1–14.

Hardy, D., Besnard, A., Latil, M., Jouvion, G., Briand, D., Thépenier, C., Pascal, Q., Guguin, A., Gayraud-Morel, B., Cavaillon, J.-M., et al. (2016). Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. PLoS ONE *11*.

Haun, C.T., Vann, C.G., Roberts, B.M., Vigotsky, A.D., Schoenfeld, B.J., and Roberts, M.D. (2019). A Critical Evaluation of the Biological Construct Skeletal Muscle Hypertrophy: Size Matters but So Does the Measurement. Front. Physiol. *10*.

Hazarika Surovi, Dokun Ayotunde O., Li Yongjun, Popel Aleksander S., Kontos Christopher D., and Annex Brian H. (2007). Impaired Angiogenesis After Hindlimb Ischemia in Type 2 Diabetes Mellitus. Circ. Res. *101*, 948–956.

Hedrick, S.M., Michelini, R.H., Doedens, A.L., Goldrath, A.W., and Stone, E.L. (2012). FOXO transcription factors throughout T cell biology. Nat. Rev. Immunol. *12*.

Hepple, R.T. (2000). Skeletal muscle: microcirculatory adaptation to metabolic demand. Med. Sci. Sports Exerc. *32*, 117.

Hermansen, L., and Wachtlova, M. (1971). Capillary density of skeletal muscle in well-trained and untrained men. J. Appl. Physiol. *30*, 860–863.

Heuslein, J.L., Meisner, J.K., Li, X., Song, J., Vincentelli, H., Leiphart, R.J., Ames, E.G., and Price, R.J. (2015). Mechanisms of Amplified Arteriogenesis in Collateral Artery Segments Exposed to Flow Direction Reversal. Arterioscler. Thromb. Vasc. Biol. *35*, 2354–2365.

Hicks, C.W., Yang, C., Ndumele, C.E., Folsom, A.R., Heiss, G., Black, J.H., Selvin, E., and Matsushita, K. (2018). Associations of Obesity With Incident Hospitalization Related to Peripheral Artery Disease and Critical Limb Ischemia in the ARIC Study. J. Am. Heart Assoc. Cardiovasc. Cerebrovasc. Dis. *7*.

van Hinsbergh, V.W.M., and Koolwijk, P. (2008). Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. Cardiovasc. Res. *78*, 203–212.

Hong, K., Zhao, G., Hong, Z., Sun, Z., Yang, Y., Clifford, P.S., Davis, M.J., Meininger, G.A., and Hill, M.A. (2016). Mechanical activation of angiotensin II type 1 receptors causes actin remodelling and myogenic responsiveness in skeletal muscle arterioles. J. Physiol. *594*, 7027–7047.

Hosaka, T., Biggs, W.H., Tieu, D., Boyer, A.D., Varki, N.M., Cavenee, W.K., and Arden, K.C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc. Natl. Acad. Sci. U. S. A. *101*, 2975–2980.

Hourdé, C., Vignaud, A., Beurdy, I., Martelly, I., Keller, A., and Ferry, A. (2006). Sustained peripheral arterial insufficiency durably impairs normal and regenerating skeletal muscle function. J. Physiol. Sci. JPS *56*, 361–367.

Ingjer, F. (1979). Capillary supply and mitochondrial content of different skeletal muscle fiber types in untrained and endurance-trained men. A histochemical and ultrastructural study. Eur. J. Appl. Physiol. *40*, 197–209.

Iruela-Arispe, M.L., Luque, A., and Lee, N. (2004). Thrombospondin modules and angiogenesis. Int. J. Biochem. Cell Biol. *36*, 1070–1078.

Ismaeel, A., Kim, J.-S., Kirk, J.S., Smith, R.S., Bohannon, W.T., and Koutakis, P. (2019). Role of Transforming Growth Factor-β in Skeletal Muscle Fibrosis: A Review. Int. J. Mol. Sci. *20*.

Ito, Y., Daitoku, H., and Fukamizu, A. (2009). Foxo1 increases pro-inflammatory gene expression by inducing C/EBPbeta in TNF-alpha-treated adipocytes. Biochem. Biophys. Res. Commun. *378*, 290–295.

Jakobsson, L., Franco, C.A., Bentley, K., Collins, R.T., Ponsioen, B., Aspalter, I.M., Rosewell, I., Busse, M., Thurston, G., Medvinsky, A., et al. (2010). Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat. Cell Biol. *12*, 943–953.

Jakubzick, C.V., Randolph, G.J., and Henson, P.M. (2017). Monocyte differentiation and antigen-presenting functions. Nat. Rev. Immunol. *17*, 349–362.

Jin Zheng-Gen, Ueba Hiroto, Tanimoto Tatsuo, Lungu Andreea O., Frame Mary D., and Berk Bradford C. (2003). Ligand-Independent Activation of Vascular Endothelial Growth Factor Receptor 2 by Fluid Shear Stress Regulates Activation of Endothelial Nitric Oxide Synthase. Circ. Res. *93*, 354–363.

Joe, A.W.B., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A., and Rossi, F.M.V. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. Nat. Cell Biol. *12*, 153–163.

Jones, D., Round, J.M., and Haan, A. de (2004). Skeletal muscle from molecules to movement: a textbook of muscle physiology for sport, exercise, physiotherapy and medicine (Edinburgh: Churchill Livingstone).

Jones, W.S., Duscha, B.D., Robbins, J.L., Duggan, N.N., Regensteiner, J.G., Kraus, W.E., Hiatt, W.R., Dokun, A.O., and Annex, B.H. (2012). Alteration in angiogenic and anti-angiogenic forms of vascular endothelial growth factor-A in skeletal muscle of patients with intermittent claudication following exercise training. Vasc. Med. Lond. Engl. *17*, 94–100.

Kierdorf, K., Prinz, M., Geissmann, F., and Perdiguero, E.G. (2015). Development and function of tissue resident macrophages in mice. Semin. Immunol. *27*, 369–378.

Kim, Y.H., Choi, J., Yang, M.J., Hong, S.P., Lee, C., Kubota, Y., Lim, D.-S., and Koh, G.Y. (2019). A MST1–FOXO1 cascade establishes endothelial tip cell polarity and facilitates sprouting angiogenesis. Nat. Commun. *10*.

Kjaer, M. (2004). Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol. Rev. *84*, 649–698.

Klotz, L.-O., Sánchez-Ramos, C., Prieto-Arroyo, I., Urbánek, P., Steinbrenner, H., and Monsalve, M. (2015). Redox regulation of FoxO transcription factors. Redox Biol. *6*, 51–72.

Kofler, S., Nickel, T., and Weis, M. (2005). Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. Clin. Sci. Lond. Engl. 1979 *108*, 205–213.

Koskinen, S.O.A., Ahtikoski, A.M., Komulainen, J., Hesselink, M.K.C., Drost, M.R., and Takala, T.E.S. (2002). Short-term effects of forced eccentric contractions on collagen synthesis and degradation in rat skeletal muscle. Pflugers Arch. *444*, 59–72.

Koutakis, P., Miserlis, D., Myers, S.A., Kim, J.K.-S., Zhu, Z., Papoutsi, E., Swanson, S.A., Haynatzki, G., Ha, D.M., Carpenter, L.A., et al. (2015). Abnormal Accumulation of Desmin in Gastrocnemius Myofibers of Patients with Peripheral Artery Disease. J. Histochem. Cytochem. *63*, 256–269.

Kozakowska, M., Pietraszek-Gremplewicz, K., Jozkowicz, A., and Dulak, J. (2015). The role of oxidative stress in skeletal muscle injury and regeneration: focus on antioxidant enzymes. J. Muscle Res. Cell Motil. *36*, 377–393.

Krishna, S.M., Moxon, J.V., and Golledge, J. (2015). A Review of the Pathophysiology and Potential Biomarkers for Peripheral Artery Disease. Int. J. Mol. Sci. *16*, 11294–11322.

Krishnasamy, K., Limbourg, A., Kapanadze, T., Gamrekelashvili, J., Beger, C., Häger, C., Lozanovski, V.J., Falk, C.S., Napp, L.C., Bauersachs, J., et al. (2017). Blood vessel control of macrophage maturation promotes arteriogenesis in ischemia. Nat. Commun. *8*, 1–14.

Laumonier, T., and Menetrey, J. (2016). Muscle injuries and strategies for improving their repair. J. Exp. Orthop. *3*.

Lemos, D.R., Babaeijandaghi, F., Low, M., Chang, C.-K., Lee, S.T., Fiore, D., Zhang, R.-H., Natarajan, A., Nedospasov, S.A., and Rossi, F.M.V. (2015). Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors. Nat. Med. *21*, 786–794.

Lepper, C., Partridge, T.A., and Fan, C.-M. (2011). An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Dev. Camb. Engl. *138*, 3639–3646.

Liang, X., Arullampalam, P., Yang, Z., and Ming, X.-F. (2019). Hypoxia Enhances Endothelial Intercellular Adhesion Molecule 1 Protein Level Through Upregulation of Arginase Type II and Mitochondrial Oxidative Stress. Front. Physiol. *10*.

Liu, T., Zhang, L., Joo, D., and Sun, S.-C. (2017). NF-κB signaling in inflammation. Signal Transduct. Target. Ther. *2*, 1–9.

Lovell, M., Harris, K., Forbes, T., Twillman, G., Abramson, B., Criqui, M.H., Schroeder, P., Mohler, E.R., and Hirsch, A.T. (2009). Peripheral arterial disease: Lack of awareness in Canada. Can. J. Cardiol. *25*, 39–45.

Lundgren, F., Dahllöf, A.G., Lundholm, K., Scherstén, T., and Volkmann, R. (1989). Intermittent claudication--surgical reconstruction or physical training? A prospective randomized trial of treatment efficiency. Ann. Surg. *209*, 346–355.

Mai, J., Virtue, A., Shen, J., Wang, H., and Yang, X.-F. (2013). An evolving new paradigm: endothelial cells – conditional innate immune cells. J. Hematol. Oncol.J Hematol Oncol *6*, 61.

Matsakas, A., Yadav, V., Lorca, S., and Narkar, V. (2013). Muscle ERRγ mitigates Duchenne muscular dystrophy via metabolic and angiogenic reprogramming. FASEB J. *27*, 4004–4016.

McDermott, M.M. (2015). LOWER EXTREMITY MANIFESTATIONS OF PERIPHERAL ARTERY DISEASE: THE PATHOPHYSIOLOGIC AND FUNCTIONAL IMPLICATIONS OF LEG ISCHEMIA. Circ. Res. *116*, 1540–1550.

Milkiewicz, M., Hudlicka, O., Brown, M.D., and Silgram, H. (2005). Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle. Am. J. Physiol. Heart Circ. Physiol. *289*, H336-343.

Monaco, C., and Paleolog, E. (2004). Nuclear factor κB: a potential therapeutic target in atherosclerosis and thrombosis. Cardiovasc. Res. *61*, 671–682.

Mondy, J.S., Lindner, V., Miyashiro, J.K., Berk, B.C., Dean, R.H., and Geary, R.L. (1997). Platelet-derived growth factor ligand and receptor expression in response to altered blood flow in vivo. Circ. Res. *81*, 320–327.

Morbidelli, L., Donnini, S., and Ziche, M. (2003). Role of nitric oxide in the modulation of angiogenesis. Curr. Pharm. Des. *9*, 521–530.

Mourkioti, F., and Rosenthal, N. (2005). IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol. *26*, 535–542.

Muller, W.A. (2014). How Endothelial Cells Regulate Transmigration of Leukocytes in the Inflammatory Response. Am. J. Pathol. *184*, 886–896.

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., et al. (1998). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J. Clin. Invest. *101*, 2567–2578.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. *13*, 9–22.

Newman, A.C., Nakatsu, M.N., Chou, W., Gershon, P.D., and Hughes, C.C.W. (2011). The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. Mol. Biol. Cell *22*, 3791–3800.

Nguyen, H.X., Lusis, A.J., and Tidball, J.G. (2005). Null mutation of myeloperoxidase in mice prevents mechanical activation of neutrophil lysis of muscle cell membranes in vitro and in vivo. J. Physiol. *565*, 403–413.

Norgren, L., Hiatt, W.R., Dormandy, J.A., Nehler, M.R., Harris, K.A., and Fowkes, F.G.R. (2007). Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur. J. Vasc. Endovasc. Surg. *33*, S1–S75.

Nwadozi, E., Roudier, E., Rullman, E., Tharmalingam, S., Liu, H.-Y., Gustafsson, T., and Haas, T.L. (2016). Endothelial FoxO proteins impair insulin sensitivity and restrain muscle angiogenesis in response to a high-fat diet. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *30*, 3039–3052.

Nwadozi, E., Rudnicki, M., De Ciantis, M., Milkovich, S., Pulbere, A., Roudier, E., Birot, O., Gustafsson, T., Ellis, C.G., and Haas, T.L. (2020). High-fat diet pre-conditioning improves microvascular remodelling during regeneration of ischaemic mouse skeletal muscle. Acta Physiol. Oxf. Engl. e13449.

Ochoa, O., Sun, D., Reyes-Reyna, S.M., Waite, L.L., Michalek, J.E., McManus, L.M., and Shireman, P.K. (2007). Delayed angiogenesis and VEGF production in CCR2−/− mice during impaired skeletal muscle regeneration. Am. J. Physiol.-Regul. Integr. Comp. Physiol. *293*, R651–R661.

Oh, I.-Y., Yoon, C.-H., Hur, J., Kim, J.-H., Kim, T.-Y., Lee, C.-S., Park, K.-W., Chae, I.-H., Oh, B.-H., Park, Y.-B., et al. (2007). Involvement of E-selectin in recruitment of endothelial progenitor cells and angiogenesis in ischemic muscle. Blood *110*, 3891–3899.

Ohno, Y., Ando, K., Ito, T., Suda, Y., Matsui, Y., Oyama, A., Kaneko, H., Yokoyama, S., Egawa, T., and Goto, K. (2019). Lactate Stimulates a Potential for Hypertrophy and Regeneration of Mouse Skeletal Muscle. Nutrients *11*.

Olfert, I.M., Howlett, R.A., Wagner, P.D., and Breen, E.C. (2010). Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. Am. J. Physiol. - Regul. Integr. Comp. Physiol. *299*, R1059–R1067.

Owens, C.D., Ho, K.J., and Conte, M.S. (2008). Lower extremity vein graft failure: a translational approach. Vasc. Med. *13*, 63–74.

Paik, J.-H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309–323.

Paoni, N.F., Peale, F., Wang, F., Errett-Baroncini, C., Steinmetz, H., Toy, K., Bai, W., Williams, P.M., Bunting, S., Gerritsen, M.E., et al. (2002). Time course of skeletal muscle repair and gene expression following acute hind limb ischemia in mice. Physiol. Genomics *11*, 263–272.

Paradis, S., Charles, A.-L., Meyer, A., Lejay, A., Scholey, J.W., Chakfé, N., Zoll, J., and Geny, B. (2016). Chronology of mitochondrial and cellular events during skeletal muscle ischemia-reperfusion. Am. J. Physiol. - Cell Physiol. *310*, C968–C982.

Peirce, S.M., and Skalak, T.C. (2003). Microvascular remodeling: a complex continuum spanning angiogenesis to arteriogenesis. Microcirc. N. Y. N 1994 *10*, 99–111.

Pellegrino, M.A., Desaphy, J.-F., Brocca, L., Pierno, S., Camerino, D.C., and Bottinelli, R. (2011). Redox homeostasis, oxidative stress and disuse muscle atrophy. J. Physiol. *589*, 2147–2160.

Penn, B.H., Bergstrom, D.A., Dilworth, F.J., Bengal, E., and Tapscott, S.J. (2004). A MyoD-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. Genes Dev. *18*, 2348–2353.

Petrov, V.V., Fagard, R.H., and Lijnen, P.J. (2002). Stimulation of Collagen Production by Transforming Growth Factor-β_1 During Differentiation of Cardiac Fibroblasts to Myofibroblasts. Hypertension *39*, 258–263.

Pietersma, A., Tilly, B.C., Gaestel, M., de Jong, N., Lee, J.C., Koster, J.F., and Sluiter, W. (1997). P38 Mitogen Activated Protein Kinase Regulates Endothelial VCAM-1 Expression at the Post-transcriptional Level. Biochem. Biophys. Res. Commun. *230*, 44–48.

Pipinos, I.I., Judge, A.R., Selsby, J.T., Zhu, Z., Swanson, S.A., Nella, A.A., and Dodd, S.L. (2007). The myopathy of peripheral arterial occlusive disease: part 1. Functional and histomorphological changes and evidence for mitochondrial dysfunction. Vasc. Endovascular Surg. *41*, 481–489.

Pipinos, I.I., Swanson, S.A., Zhu, Z., Nella, A.A., Weiss, D.J., Gutti, T.L., McComb, R.D., Baxter, B.T., Lynch, T.G., and Casale, G.P. (2008). Chronically ischemic mouse skeletal muscle exhibits myopathy in association with mitochondrial dysfunction and oxidative damage. Am. J. Physiol. Regul. Integr. Comp. Physiol. *295*, R290-296.

Pittman, R.N. (2011). Oxygen Gradients in the Microcirculation. Acta Physiol. Oxf. Engl. *202*, 311–322.

Pober, J.S., and Sessa, W.C. (2015). Inflammation and the Blood Microvascular System. Cold Spring Harb. Perspect. Biol. *7*.

Potente, M., and Carmeliet, P. (2017). The Link Between Angiogenesis and Endothelial Metabolism. Annu. Rev. Physiol. *79*, 43–66.

Potente, M., Urbich, C., Sasaki, K., Hofmann, W.K., Heeschen, C., Aicher, A., Kollipara, R., DePinho, R.A., Zeiher, A.M., and Dimmeler, S. (2005). Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J. Clin. Invest. *115*, 2382–2392.

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and Therapeutic Aspects of Angiogenesis. Cell *146*, 873–887.

Rice, T.W., and Lumsden, A.B. (2006). Optimal Medical Management of Peripheral Arterial Disease. Vasc. Endovascular Surg. *40*, 312–327.

Richards, J., Gabunia, K., Kelemen, S.E., Kako, F., Choi, E.T., and Autieri, M.V. (2015). Interleukin-19 increases Angiogenesis in ischemic hind limbs by Direct Effects on both Endothelial Cells and Macrophage Polarization. J. Mol. Cell. Cardiol. *79*, 21–31.

Richardson, R.S., Grassi, B., Gavin, T.P., Haseler, L.J., Tagore, K., Roca, J., and Wagner, P.D. (1999). Evidence of O_2 supply-dependent$\dot{V}O_{2max}$ in the exercise-trained human quadriceps. J. Appl. Physiol. *86*, 1048–1053.

Robinson, W.P., Nguyen, L.L., Bafford, R., and Belkin, M. (2011). Results of second-time angioplasty and stenting for femoropopliteal occlusive disease and factors affecting outcomes. J. Vasc. Surg. *53*, 651–657.

Roudier, E., Milkiewicz, M., Birot, O., Slopack, D., Montelius, A., Gustafsson, T., Paik, J.H., DePinho, R.A., Casale, G.P., Pipinos, I.I., et al. (2013). Endothelial FoxO1 is an intrinsic regulator of thrombospondin 1 expression that restrains angiogenesis in ischemic muscle. Angiogenesis *16*.

Rudnicki, M., Abdifarkosh, G., Nwadozi, E., Ramos, S.V., Makki, A., Sepa-Kishi, D.M., Ceddia, R.B., Perry, C.G., Roudier, E., and Haas, T.L. (2018). Endothelial-specific FoxO1 depletion prevents obesity-related disorders by increasing vascular metabolism and growth. ELife *7*.

Rutherford, R.B., Baker, J.D., Ernst, C., Johnston, K.W., Porter, J.M., Ahn, S., and Jones, D.N. (1997). Recommended standards for reports dealing with lower extremity ischemia: Revised version. J. Vasc. Surg. *26*, 517–538.

Salih, D.A.M., and Brunet, A. (2008). FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr. Opin. Cell Biol. *20*, 126–136.

Sambasivan, R., Yao, R., Kissenpfennig, A., Van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., Guenou, H., Malissen, B., Tajbakhsh, S., and Galy, A. (2011). Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. Dev. Camb. Engl. *138*, 3647–3656.

Sang, Q.X.A. (1998). Complex role of matrix metalloproteinases in angiogenesis. Cell Res. *8*, 171–177.

Sarkar, S., Vellaichamy, E., Young, D., and Sen, S. (2004). Influence of cytokines and growth factors in ANG II-mediated collagen upregulation by fibroblasts in rats: role of myocytes. Am. J. Physiol.-Heart Circ. Physiol. *287*, H107–H117.

Schieber, M.N., Hasenkamp, R.M., Pipinos, I.I., Johanning, J.M., Stergiou, N., DeSpiegelaere, H.K., Chien, J.H., and Myers, S.A. (2017). Muscle strength and control characteristics are altered by peripheral artery disease. J. Vasc. Surg. *66*, 178-186.e12.

Scholz, D., Ziegelhoeffer, T., Helisch, A., Wagner, S., Friedrich, C., Podzuweit, T., and Schaper, W. (2002). Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. J. Mol. Cell. Cardiol. *34*, 775–787.

Sengupta, A., Chakraborty, S., Paik, J., Yutzey, K.E., and Evans-Anderson, H.J. (2012). FoxO1 is required in endothelial but not myocardial cell lineages during cardiovascular development. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *241*, 803–813.

Sheng, Y., and Zhu, L. (2018). The crosstalk between autonomic nervous system and blood vessels. Int. J. Physiol. Pathophysiol. Pharmacol. *10*, 17–28.

Slopack, D., Roudier, E., Liu, S.T.K., Nwadozi, E., Birot, O., and Haas, T.L. (2014). Forkhead BoxO transcription factors restrain exercise-induced angiogenesis. J. Physiol. *592*, 4069–4082.

Sternbergh, C. (1992). The temporal relationship between endothelial cell dysfunction and skeletal muscle damage after ischemia and reperfusion. *16*, 10.

Sunderkötter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R., and Sorg, C. (1994). Macrophages and angiogenesis. J. Leukoc. Biol. *55*, 410–422.

Takeda, Y., Costa, S., Delamarre, E., Roncal, C., Leite de Oliveira, R., Squadrito, M.L., Finisguerra, V., Deschoemaeker, S., Bruyère, F., Wenes, M., et al. (2011). Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature *479*, 122–126.

Takeshita, S., Weir, L., Chen, D., Zheng, L.P., Riessen, R., Bauters, C., Symes, J.F., Ferrara, N., and Isner, J.M. (1996). Therapeutic Angiogenesis Following Arterial Gene Transfer of Vascular Endothelial Growth Factor in a Rabbit Model of Hindlimb Ischemia. Biochem. Biophys. Res. Commun. *227*, 628–635.

Tan, H.-Y., Wang, N., Li, S., Hong, M., Wang, X., and Feng, Y. (2016). The Reactive Oxygen Species in Macrophage Polarization: Reflecting Its Dual Role in Progression and Treatment of Human Diseases. Oxid. Med. Cell. Longev. *2016*.

Thomas, K., Engler, A.J., and Meyer, G.A. (2015). Extracellular matrix regulation in the muscle satellite cell niche. Connect. Tissue Res. *56*, 1–8.

Tidball, J.G. (2011). Mechanisms of Muscle Injury, Repair, and Regeneration. In Comprehensive Physiology, R. Terjung, ed. (Hoboken, NJ, USA: John Wiley & Sons, Inc.), p. c100092.

Tidball, J.G. (2017). Regulation of muscle growth and regeneration by the immune system. Nat. Rev. Immunol. *17*, 165–178.

Tidball, J.G., and Villalta, S.A. (2010). Regulatory interactions between muscle and the immune system during muscle regeneration. Am. J. Physiol. - Regul. Integr. Comp. Physiol. *298*, R1173–R1187.

Timmerman, K.L., Lee, J.L., Dreyer, H.C., Dhanani, S., Glynn, E.L., Fry, C.S., Drummond, M.J., Sheffield-Moore, M., Rasmussen, B.B., and Volpi, E. (2010). Insulin stimulates human skeletal muscle protein synthesis via an indirect mechanism involving endothelial-dependent vasodilation and mammalian target of rapamycin complex 1 signaling. J. Clin. Endocrinol. Metab. *95*, 3848–3857.

Tojo, T., Ushio-Fukai, M., Yamaoka-Tojo, M., Ikeda, S., Patrushev, N., and Alexander, R.W. (2005). Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. Circulation *111*, 2347–2355.

Toumi, H., F'guyer, S., and Best, T.M. (2006a). The role of neutrophils in injury and repair following muscle stretch. J. Anat. *208*, 459–470.

Toumi, H., Hegge, J., Subbotin, V., Noble, M., Herweijer, H., Best, T.M., and Hagstrom, J.E. (2006b). Rapid intravascular injection into limb skeletal muscle: a damage assessment study. Mol. Ther. J. Am. Soc. Gene Ther. *13*, 229–236.

Trensz, F., Lucien, F., Couture, V., Söllrald, T., Drouin, G., Rouleau, A.-J., Grandbois, M., Lacraz, G., and Grenier, G. (2015). Increased microenvironment stiffness in damaged myofibers promotes myogenic progenitor cell proliferation. Skelet. Muscle *5*, 5.

Tronc, F., Wassef, M., Esposito, B., Henrion, D., Glagov, S., and Tedgui, A. (1996). Role of NO in flow-induced remodeling of the rabbit common carotid artery. Arterioscler. Thromb. Vasc. Biol. *16*, 1256–1262.

Tsai, S., Hollenbeck, S.T., Ryer, E.J., Edlin, R., Yamanouchi, D., Kundi, R., Wang, C., Liu, B., and Kent, K.C. (2009). TGF-β through Smad3 signaling stimulates vascular smooth muscle cell proliferation and neointimal formation. Am. J. Physiol.-Heart Circ. Physiol. *297*, H540–H549.

Tuma, R.F., Durán, W.N., and Ley, K. (2008). Handbook of physiology a critical, comprehensive presentation of physiological knowledge and concepts. Sect. 2, The cardiovascular system Vol. 4, Microcirculation. Sect. 2, The cardiovascular system Vol. 4, Microcirculation. (Bethesda, Md: American Physiolog. Society).

Uchida, C., Nwadozi, E., Hasanee, A., Olenich, S., Olfert, I.M., and Haas, T.L. (2015). Muscle-derived vascular endothelial growth factor regulates microvascular remodelling in response to increased shear stress in mice. Acta Physiol. *214*, 349–360.

Velasco-Hernandez, T., Säwén, P., Bryder, D., and Cammenga, J. (2016). Potential Pitfalls of the Mx1-Cre System: Implications for Experimental Modeling of Normal and Malignant Hematopoiesis. Stem Cell Rep. *7*, 11–18.

Vogt, P.K., Jiang, H., and Aoki, M. (2005). Triple Layer Control: Phosphorylation, Acetylation and Ubiquitination of FOXO Proteins. Cell Cycle *4*, 908–913.

Wang, Y.X., and Rudnicki, M.A. (2011). Satellite cells, the engines of muscle repair. Nat. Rev. Mol. Cell Biol. *13*, 127–133.

Wang, H., Melton, D.W., Porter, L., Sarwar, Z.U., McManus, L.M., and Shireman, P.K. (2014a). Altered macrophage phenotype transition impairs skeletal muscle regeneration. Am. J. Pathol. *184*, 1167–1184.

Wang, Y., Zhou, Y., and Graves, D.T. (2014b). FOXO Transcription Factors: Their Clinical Significance and Regulation. BioMed Res. Int. *2014*.

Ward Michael R., Pasterkamp Gerard, Yeung Alan C., and Borst Cornelius (2000). Arterial Remodeling. Circulation *102*, 1186–1191.

Warren, G.L., Summan, M., Gao, X., Chapman, R., Hulderman, T., and Simeonova, P.P. (2007). Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. J. Physiol. *582*, 825–841.

Webb, A.E., and Brunet, A. (2014). FOXO transcription factors: key regulators of cellular quality control. Trends Biochem. Sci. *39*, 159–169.

Whitehead, N.P., Yeung, E.W., and Allen, D.G. (2006). Muscle damage in mdx (dystrophic) mice: role of calcium and reactive oxygen species. Clin. Exp. Pharmacol. Physiol. *33*, 657–662.

Wieteska-Skrzeczyńska, W., Grzelkowska-Kowalczyk, K., Tokarska, J., and Grabiec, K. (2011). Growth factor and cytokine interactions in myogenesis. Part I. The effect of TNF-alpha and IFN-gamma on IGF-I-dependent differentiation in mouse C2C12 myogenic cells. Pol. J. Vet. Sci. *14*, 417–424.

Wilhelm, K., Happel, K., Eelen, G., Schoors, S., Oellerich, M.F., Lim, R., Zimmermann, B., Aspalter, I.M., Franco, C.A., Boettger, T., et al. (2016). FOXO1 couples metabolic activity and growth state in the vascular endothelium. Nature *529*, 216–220.

Wittchen, E.S. (2009). Endothelial signaling in paracellular and transcellular leukocyte transmigration. Front. Biosci. J. Virtual Libr. *14*, 2522–2545.

Wong, J., Bennett, W., Ferguson, M.W.J., and McGrouther, D.A. (2006). Microscopic and histological examination of the mouse hindpaw digit and flexor tendon arrangement with 3D reconstruction. J. Anat. *209*, 533–545.

Wozniak, A.C., Kong, J., Bock, E., Pilipowicz, O., and Anderson, J.E. (2005). Signaling satellite-cell activation in skeletal muscle: markers, models, stretch, and potential alternate pathways. Muscle Nerve *31*, 283–300.

Wu, L.-W., Mayo, L.D., Dunbar, J.D., Kessler, K.M., Baerwald, M.R., Jaffe, E.A., Wang, D., Warren, R.S., and Donner, D.B. (2000). Utilization of Distinct Signaling Pathways by Receptors for Vascular Endothelial Cell Growth Factor and Other Mitogens in the Induction of Endothelial Cell Proliferation. J. Biol. Chem. *275*, 5096–5103.

Wynn, T.A., and Vannella, K.M. (2016). Macrophages in tissue repair, regeneration, and fibrosis. Immunity *44*, 450–462.

Yablonka-Reuveni, Z., and Rivera, A.J. (1997). Influence of PDGF-BB on Proliferation and Transition Through the MyoD-myogenin-MEF2A Expression Program During Myogenesis in Mouse C2 Myoblasts. Growth Factors *15*, 1–27.

Yang, J., Zhang, L., Yu, C., Yang, X.-F., and Wang, H. (2014). Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark. Res. *2*, 1.

Yin, H., Price, F., and Rudnicki, M.A. (2013). Satellite Cells and the Muscle Stem Cell Niche. Physiol. Rev. *93*, 23–67.

Yu, J., and Dardik, A. (2018). A Murine Model of Hind Limb Ischemia to Study Angiogenesis and Arteriogenesis. Methods Mol. Biol. Clifton NJ *1717*, 135–143.

Zhang, S., Zhao, Y., Xu, M., Yu, L., Zhao, Y., Chen, J., Yuan, Y., Zheng, Q., and Niu, X. (2013). FoxO3a Modulates Hypoxia Stress Induced Oxidative Stress and Apoptosis in Cardiac Microvascular Endothelial Cells. PLoS ONE *8*, e80342.

Zhang, X., Tang, N., Hadden, T.J., and Rishi, A.K. (2011). Akt, FoxO and regulation of apoptosis. Biochim. Biophys. Acta BBA - Mol. Cell Res. *1813*, 1978–1986.