REGULATION OF EXERCISE INDUCED ENDOTHELIAL SPROUT FORMATION

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Abstract

Capillary sprouting is known to be guided by Dll4/Notch signaling in mouse retina, while maturation of the endothelium is regulated by Dll1 and Tie2. This study investigates the key molecules involved in endothelial sprouting and maturation, and the gene that orchestrates the expression of these targets in skeletal muscle in response to exercise. In exercised mice, Dll1 and Dll4 proteins were decreased with repeated training. Tie2 mRNA was downregulated with 5 days of exercise. The suppression of these molecules may induce destabilization of the endothelium and allow for sprouting to occur. Moreover, FoxO transcription factors have been shown to be anti-angiogenic and may negatively regulate genes involved in sprouting. Both Dll1 and Dll4 expression were not altered with repeated exercise in mice with endothelial cell directed conditional deletion of FoxO1/3a/4 ($FoxO^{\Delta}$). We have provided insight into the mechanisms behind the initiation of capillary growth in skeletal muscle induced by exercise.

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This thesis is dedicated to my parents, my sister, and Kristina. Thank you for your everlasting love, patience, and encouragement. I would not be where I am today without your support.

The ten thousand mile journey continues…

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List of Abbreviations

3D – Three dimensional (type-1 collagen matrix)
Akt – Protein kinase B
Ang1 – Angiopoietin 1
Ang1 – Angiopoietin 2
ARNT – Arylhydrocarbon nuclear receptor
ATP – Adenosine triphosphate
Dll – Delta-like ligand
Dll1 – Delta-like ligand 1
Dll4 – Delta-like ligand 4
EC – Endothelial cell
EDL – Extensor digitorum longus
eNOS- Endothelial nitric oxide synthase
Fox - Forkhead
FoxO – Forkhead box “O”
$FoxO^{\Delta}$ – MxCre+:$FoxO1,3,4^{L/L}$
$FoxO^{L/L}$ – MxCre-:FoxO1,3,4
HIF – Hypoxia inducible factor
HIF-1α – Hypoxia inducible factor 1 alpha
HIF-1β – Hypoxia inducible factor 1 beta
HRE – Hypoxia responsive elements
MMP-2 – Matrix metalloproteinase 2
MMP-9 – Matrix metalloproteinase 9
mRNA – Messenger ribonucleic acid
MT1-MMP – Membrane type 1 metalloproteinase
NAPDH – Nicotinamide adenine dinucleotide phosphate
NRP – Neuropilin
Pdgfb – Platelet-derived growth factor “b”
PI3K – Phosphoinositide 3-kinase
Sema – Semaphorin
Sema3 – Semaphorin class 3
Sema3F – Semaphorin class 3F
Tie2 – Endothelial cell specific tyrosine kinase receptor
TSP-1- Thrombospondin-1
TSR1-3 – Thrombospondin-1 type-1 repeats
VASH-1 – Vasohibin-1
VEGF – Vascular endothelial growth factor
VEGFR1 – Vascular endothelial growth factor receptor 1
VEGFR2 – Vascular endothelial growth factor receptor 2
VEGFR3 – Vascular endothelial growth factor receptor 3

Chapter 1: Literature Review

1.1 Skeletal Muscle

Skeletal muscle is a type of striated muscle that represents approximately 49% of the total body mass in men and 41% of the total body mass in women (Cattrysse et al., 2002). It is a malleable tissue that can adapt to changes in functional demands with its mechanical and metabolic properties (Flück, 2006). These changes include the alteration of muscular structures to enhance contractile force, velocity, and endurance. Muscular adaptions are crucial in regulating homeostasis within the body. Muscle loading and contraction are known to be the primary stimuli for muscular adaptions (Flück & Hoppeler, 2003). A prime example that contributes to this phenomenon is physical exercise.

1.2 Exercise

It is well recognized that regular physical activity is necessary for attaining and maintaining optimal health. The physiological adaption elicited by physical activity is largely dependent on the intensity, duration, frequency, and the type of exercise being executed. A single bout of exercise is sufficient to induce molecular synthesis and degradation within our body (Booth & Thomason, 1991). Fibre type switching, and increase in mitochondrion protein expression and anti-oxidants are some of the ways in which skeletal muscles adapt to prolonged or repeated exercise (Flück & Hoppeler, 2003; Powers & Jackson, 2008).

Aerobic exercise training is strongly associated with cardiovascular adaptations, such as increase in cardiac output to improve blood flow and oxidative capacity (Booth & Thomason, 1991). Vascular remodeling also occurs with endurance exercise by increasing the number of capillaries in the working tissues to allow for a greater substrate exchange area (Bloor, 2005; Egginton, 2009; Lloyd et al., 2003).

Muscular adaptations that occur with changes in the environment enable the muscle to fulfill the requirements of the body, and to minimize disruption of homeostasis which permits the organism to endure longer duration of physical work at the same power output before fatigue (Powers & Jackson, 2008).

1.3 Capillaries

Capillaries are the smallest microvessels in the circulation. They help maintain tissue viability by facilitating in the delivery of crucial nutrients, metabolites, and respiratory gases. A blood capillary consists of a lumen lined with a monolayer of endothelial cells (Hwa & Aird, 2007). The endothelial cells are attached adjacent to one another, embedded in the basement membrane of the extracellular matrix. This layer of sub-endothelial basement membrane regulates the exchange between the bloodstream and the surrounding tissues. Mural cells such as pericytes wrap around the capillaries, and help regulate endothelial proliferation and differentiation (Shepro & Morel, 1993).

Capillaries are crucial for oxygen delivery and removal of metabolites from myofibres, particularly during periods of increased metabolic demand (Egginton & Gaffney, 2010). Adequate tissue perfusion is required for proper functioning of working tissue, as perfusion/metabolism mismatch could lead to insufficient production of ATP

and the increased accumulation of metabolic waste products (Wagner, 2000). The capillaries within the skeletal muscle run parallel to the muscle fibres, forming a network which supplies the tissue with adequate oxygen to maintain its functions (Egginton, 2011; Krogh, 1919a). The amount of blood vessels that surrounds a myofibre could determine the efficiency of tissue oxygenation. Plasticity within the microvascular network allows for changes in capillary density by the process of capillary growth (angiogenesis); this process commences according to the metabolic need of the surrounding tissue (Egginton, 2011). Increasing muscle capillarization can 1) increase the surface area for respiratory gas and substrate/metabolite exchange 2) decrease average diffusion path length within the muscle 3) increase the length of time for diffusive exchange between blood and tissue (Bloor, 2005). The early work of August Krogh showed the density of capillaries in the muscle is proportionate to the basal metabolic rate of the animal, quantified by measuring the number of capillaries per unit area of transverse muscle cross-section (Krogh, 1919a, 1919b). This technique is still commonly used by investigators to study vessel growth and regression.

1.3.1 Fibre type specific capillarization

The amount of capillarization within myofibres varies between the different fibre types. Slow-oxidative type 1 fibres are surrounded by more capillaries since they contain large numbers of oxidative enzymes with a large capacity for aerobic metabolism (Carrow et al., 1967; Gray & Renkin, 1978). In contrast, fast-twitch type IIb/x fibres have a relatively low mitochondrial volume and a limited capacity for aerobic metabolism, thus they require fewer capillaries (Gray & Renkin, 1978; Hudlická, 1985).

Alterations in fibre types and metabolic activity could lead to changes in blood vessel density as an adaptive response of the tissue (Hudlická et al., 1982). The processes that permit these physiological changes are driven by various cell signaling pathways, which will be described in detail in the following sections.

1.4 Angiogenesis

1.4.1 Angiogenesis

Angiogenesis is the physiological process that involves the growth of new capillaries from pre-existing blood vessels. It is a vital process that occurs during growth and development, and it is sustained and regulated throughout life. Excess or insufficient capillary growth can lead to pathological conditions, such as tumor growth or peripheral artery disease (Carmeliet, 2003). When an angiogenic stimulus is present, endothelial cells from existing small vessels undergo proliferation, migration, differentiation, cell survival, and specialization to develop new blood vessels (Gerhardt, 2008). The emerging vessels may come together or divide to form a capillary network that can supply a greater area within the tissue to match the changes in metabolic demands. The sustainability and growth of the network is tightly regulated by the balance between pro- and anti-angiogenic factors. The continual opposition of these regulators and the downstream signaling pathways that they trigger will determine whether or not angiogenesis occurs within the body.

1.4.2 Exercise induced angiogenesis

Exercise is a powerful stimulus for structural remodeling of the vasculature (Prior et al., 2004). During resting metabolic state, blood flow is greater in muscles with a higher quantity of oxidative fibres, but when muscle contraction occurs, vasodilators secreted by endothelial cells further increase blood flow to ensure a close coupling between muscle oxygen delivery and metabolic demand (Egginton & Hudlická, 1999; Hudlická, 1998). The initiation of vessel growth is dependent on the intensity of training, and it exhibits a much greater response in animals trained by running to exhaustion compared to moderate intensity exercise (Waters et al., 2004). The increase in muscle capillary would optimize the distribution of blood within the working tissue, and enhanced blood-tissue exchange properties (Bloor, 2005). Muscle samples obtained by needle biopsies have revealed that the number of capillaries per muscle fibre is higher in well-trained athletes compared to sedentary adults (Hermansen & Wachtlova, 1971). Endurance exercise performed at near maximal aerobic capacity enhances capillary-to-fibre ratio in active skeletal muscle as early as 14 days of training (Slopack et al., manuscript in progress). When exercise training ceases, it can cause capillary regression as a consequence of detraining induced decreases in metabolic demand (Malek et al., 2010). This reduction in vascular density can occur as rapidly as neovascularisation following exercise (Malek et al., 2010; Roudier et al., 2010). These adaptations exhibited by trained muscle have been demonstrated in numerous studies conducted on various mammalians (Prior et al., 2004).

1.4.3 Forms of angiogenesis

Angiogenesis can occur in two ways, sprouting angiogenesis and intussusception, commonly known as splitting angiogenesis. Both mechanisms lead to the expansion of the capillary network, but they involve different structural organization and signaling molecules.

1.4.4 Splitting angiogenesis

Intussusceptive microvascular growth is a non-sprouting form of angiogenesis (Kurz et al., 2003). It was first described by Caduff and colleagues when they observed the rapidly developing microvasculature in the postnatal rat lung (Caduff et al., 1986). Unlike angiogenic sprouting, intussusceptive progression does not rely on the proliferation and migration of endothelial cells (Egginton et al., 2001; Williams et al., 2006). The occurrence of vessel splitting is evident by the presence of transcapillary (intraluminal) tissue pillars (Burri & Tarek, 1990; Caduff et al., 1986). This process commences with the projection of opposing capillary walls into the vessel lumen, followed by the formation of a contact zone between the endothelial cells (Burri & Tarek, 1990). Once the interendothelial junction is established, the endothelial bilayer becomes perforated centrally and transluminal pillar is formed, such pillars range from 1 to 2.5um in diameter (Djonov et al., 2003; Kurz et al., 2003). Intussusceptive angiogenesis plays a major role in vascular development, and recent studies have identified intravascular mechanical stimuli, such as shear stress that can initiate this process.

1.4.5 Sprouting angiogenesis

When an angiogenic stimulus triggers the activation of endothelial cell, proteases are released and induce the enzymatic degradation of the capillary basement (Haas, 2005). This allows endothelial cells to proliferate and migrate from the vessel wall into the interstitial matrix to form new connections with neighboring vessels. The sprouting process is highly regulated, as a distinct site must be selected from the original blood vessel in order for sprout formation to transpire (Gerhardt, 2008). The developing capillary sprout will protrude through the extracellular matrix toward an angiogenic stimulus, such as VEGF. Each sprout is led by a motile tip cell (Napp et al., 2012). The filopodia of the endothelial tip are enriched with VEGF receptors, allowing them to guide the developing sprout towards the VEGF source (Jakobsson et al., 2010). Furthermore, the filopodia of the tip cell are enriched with basement membrane proteolytic enzyme, membrane type 1 metalloproteinase (MT1-MMP) that facilitates migration toward the VEGF gradient (van Hinsbergh & Koolwijk, 2008). Following behind a tip cell are proliferating endothelial stalk cells that cause the elongation of the capillary sprout, and eventually become the trunk of the newly formed vessel (Blanco & Gerhardt, 2012). Tip cells are frequently identified by Platelet-derived growth factor “b” (Pdgfb) and VEGFR3 in the mouse embryo and retinal vasculature (Hellström et al., 2007; Tammela et al., 2008). The association of these proteins with a tip cell phenotype allows researchers to distinguish between filopodia and stalk cells when observing endothelial sprouts.

1.4.6 Tip cell and stalk cell selection

The selection and arrangement of stalk and tip cells in the endothelium is accomplished by Dll4/Notch mediated lateral inhibition (Blanco & Gerhardt, 2012). Much of the literature that defined the effects of Notch/Dll signaling in endothelial sprouts is based on studies of the retinal vasculatures and the zebrafish embryo. The signaling mechanism behind tip cell formation has never been examined in complex tissues, like skeletal muscle. The Notch signaling pathway regulates tip cell formation during angiogenic sprouting, as shown in figure 1. It is a cell-cell signaling pathway that is activated by the binding of Notch receptors to the Delta-like ligand 4 (Dll4) on neighbouring cells (Kume, 2012). When Dll4 expression is upregulated by VEGF signaling in tip cells, it leads to the activation of Notch receptors in stalk cells, and in turn down-modulates VEGFR2/3 and upregulates VEGFR1 (Blanco & Gerhardt, 2012). The increase of VEGFR1 sequesters VEGF away from VEGFR-2, and results in the suppression of tip cell phenotype in the neighbouring (stalk) cells due to the decrease in VEGF induced migratory response (Blanco & Gerhardt, 2012; Carmeliet et al., 2009). The interaction between VEGF and Dll4-Notch signaling facilitates the functional distinction of endothelial cells into tip or stalk cells (Gerhardt, 2008).

Recent time-lapse confocal imaging revealed that the tip/stalk cell selection is a dynamic process, as endothelial cells along the extending sprout compete for the tip position (Jakobsson et al., 2010). The position shuffling that occurs between tip cells and stalk cells is coordinated by VEGF and Notch activities. Fluctuation of VEGFR2 may occur during endothelial outgrowth, and cells with higher VEGFR2 and lower VEGFR1 expression will surpass adjacent cells (Jakobsson et al., 2010; Siekmann et

al., 2013). This allows stalk cell with higher VEGFR2 levels to move into the tip position, and subsequently activates the Dll4/Notch pathway and suppresses its neighbouring cells from becoming tip cells (Jakobsson et al., 2010; Siekmann et al., 2013). Cell shuffling is hypothesized to enhance the ability for endothelial cells to determine the direction of VEGF gradient, hence providing a more robust network formation (Geudens & Gerhardt, 2011).

Another Delta-like ligand, Dll1, is also expressed in the vascular endothelium (Kume, 2009). It is largely known to regulate vascular stability and the maintenance of arterial phenotype (Limbourg et al., 2007). It has been recently identified as a key player in tip cell selection in mouse retina (Napp et al., 2012).

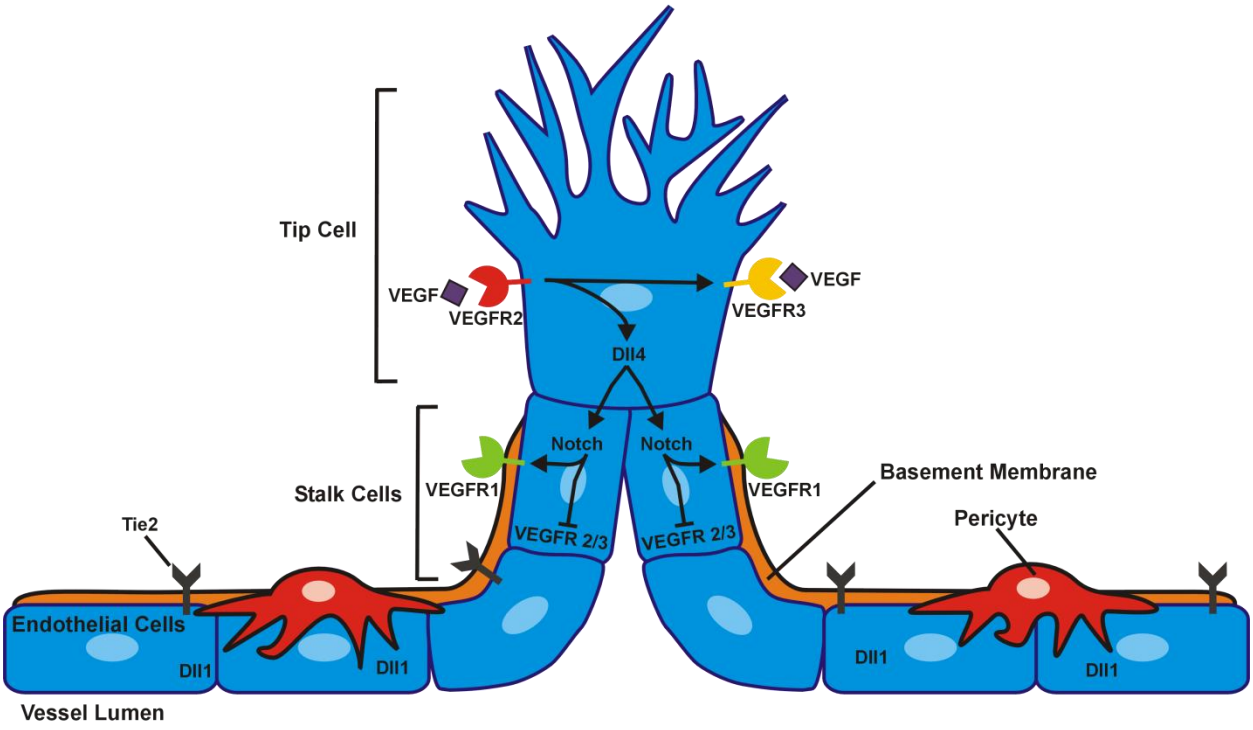


Figure 1-1. Dll4/Notch signaling mediate tip and stalk cell selection

Dll4/Notch signaling mediates the selection and arrangement of stalk and tip cells through lateral inhibition in retinal vasculature and zebrafish embryo. VEGF signaling upregulates Dll4 expression, which activates Notch receptors in stalk cells, and leads to the suppression of VEGR2/3 and upregulation of VEGFR1. This limits VEGF binding to VEGFR2/3, and results in the inhibition of tip cell phenotype in the neighbouring stalk cells.

1.5 Stimulation of angiogenesis

1.5.1 Mechanical stretch

Mechanical stretch in muscle is associated with sprouting angiogenesis. Muscle sarcomere stretch can lead to endothelial stretch as external elastic force is applied to capillaries through the connection to skeletal muscle via connective tissue and extracellular matrix (Brown & Hudlicka, 2003). Synergistic muscle ablation is an

experimental model of muscle stretch and overload. Contraction of skeletal muscle in this manner can increase VEGF mRNA and protein production in vivo (Rivilis et al., 2002), and upregulate Ang2 and Tie expression in vitro (Chang et al., 2003). Increased MMP-2 and MT1-MMP levels have also been observed in overloading the extensor digitorum longus (EDL) muscle in rats (Rivilis et al., 2002). These actions guided by mechanical forces developed within active muscle are stimuli that promote sprouting angiogenesis.

1.5.2 Hypoxia

Similar to other bodily tissues, resting skeletal muscle homeostasis is compromised under acute or chronic hypoxic exposure, and the implementation of exercise could increase the magnitude of this imbalance (Lundby et al., 2009). Oxygen availability can drop in the transition from rest to exercise during normoxic conditions, suggesting that exercising skeletal muscle operates at a very low partial pressure of oxygen (Richardson et al., 1995). The hypoxic stimulus brings forth inadequate oxygen delivery/availability at the tissue level, thus, the tissue demand exceeds its oxygen supply. All nucleated cells in the human body have the ability to sense oxygen and are able to respond to oxygen shortage in order to maintain homeostasis (Lundby et al., 2009). The key mediator of cellular hypoxia is the hypoxia inducible factor (HIF) pathway, discovered by Semenza and colleagues (Wang & Semenza, 1995). HIF-1 is a DNA-binding protein that is composed of two subunits: HIF-1α, which is highly sensitive to oxygen and has a short half-life, and HIF-1β (or ARNT: aryl hydrocarbon nuclear receptor) which is far less sensitive to oxygen levels (Semenza, 1999). HIF-1α is

degraded through hydroxylation under normoxic conditions (Lundby et al., 2009). During hypoxia, HIF-1α proteasomal degradation is blocked due to the inhibition of hydroxylation, and results in the accumulation of HIF-1α protein (Lundby et al., 2009). This allows for HIF-1α binding to HIF-1β, which forms the HIF-1 complex that can recognize hypoxia responsive elements (HRE) located in the nucleus of target genes (Lundby et al., 2009). The active HIF-1 transcriptional complex triggers expression of hundreds of downstream genes that enable the cells to manage oxygen stress, including those that regulate cell survival, metabolism, and angiogenesis (Fraisl et al., 2009).

In the vasculature, oxygen availability can also dictates whether angiogenesis occurs (Ward, 2008). Endothelial cells have a number of oxygen-sensing mechanisms, including oxygen-sensitive NADPH oxidases, endothelial nitric oxide synthase (eNOS), and heme oxygenases (Ward, 2008). The ability for endothelial cells to rapidly divide, migrate, and form new capillaries under the condition of stress and hypoxia is due to their phenotypic plasticity, and their capability to generate adequate energy for the biosynthesis of macromolecules needed for rapid cell proliferation (Fraisl et al., 2009).

1.5.3 Regulation of capillary growth by metabolism

During physical activity, increased skeletal muscle metabolism is required to match the energy requirements of the muscle. The upsurge of metabolites generated by the increase in metabolic activity may play a role in angiogenesis. Adenosine, produced from adenosine triphosphate (ATP) during exercise can elicit the angiogenic response by inducing endothelial cell migration and proliferation, and increases VEGF expression

(Adair, 2004; Grant et al., 1999; Murray & Wilson, 2001). Lactic acid, a metabolic by-product of exercise has also been shown to induce migration and proliferation of endothelial cell both in vivo and in vitro (Murray & Wilson, 2001).

1.6 Regulators of angiogenesis

1.6.1 VEGF

VEGF-A is a 35 to 45 kDa peptide growth factor (Egginton, 2009). It was first described as a potent mitogen for endothelial cells and a vascular permeability factor that is important for normal vessel growth (Ferrara & Davis-Smyth, 1997). VEGF165 is the most abundant of numerous isoforms of VEGF-A (Ferrara & Davis-Smyth, 1997). It is produced by endothelial cells, perivascular cells, and host tissue cells, such as skeletal muscle (Egginton, 2009). Stimulation of endothelial cells with VEGF *in vitro* has been shown to accelerate proliferation and migration (Gerhardt, 2008). The formation of an extracellular VEGF gradient is necessary in order for proper vascular patterning to occur. Tissue VEGF levels are regulated at the level of transcription, isoform splicing, cell surface retention, and through uptake and degradation of VEGF protein (Gerhardt, 2008). VEGF elicits angiogenic effects by binding to vascular endothelial growth factor receptor 2 (VEGFR2) located on the endothelial cell surface (Conway et al., 2001). When VEGF binds to VEGFR2 on the cell surface, it triggers a cascade of pro-angiogenic events through multiple downstream signaling pathways including phosphatidyl-inositol 3 kinase (PI3K)/Protein kinase B (Akt), RhoGTPase, mitogen activated protein kinases (MAPK), extracellular-regulated kinase (ERK), and p38 and c-

jun N-terminal kinase (JNK) (Milkiewicz, Ispanovic, Doyle, & Haas, 2006). Exercise stimuli have been shown to upregulate muscle VEGF expression in a time dependent manner (Breen et al., 1996; Gerhardt et al., 2003; Gustafsson et al., 2002; Hudlicka & Brown, 2009; Lloyd et al., 2003; Olenich et al., 2013; Waters et al., 2004).

1.6.2 Angiopoietin/Tie2

Tie2 is a member of the tyrosine kinase (RTK) family of receptors; it consists of an N-terminal ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Peters et al., 2004). Its expression is restricted to the surface of vascular endothelial cells (Schnürch & Risau, 1993). Tie2 is highly conserved across vertebrate species, from zebrafish to human, with the utmost amino acid homology found in the kinase domain (Peters et al., 2004). Disruption of the Tie2 signaling pathway in transgenic mice resulted in vessel abnormalities which led to embryonic lethality (Dumont et al., 1994; Sato et al., 1995). The blood vessels of Tie2 deficient embryos have a reduced number of endothelial cells, pericytes, and smooth muscle cells, which suggests its role in vascular branching and vessel stabilization (Patan, 1998; Peters et al., 2004).

The Tie2 receptor has 2 major ligands, angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) (Asahara et al., 1998; Schnürch & Risau, 1993). When Tie2 is bound by Ang1 it becomes activated through auto-phosphorylation, inducing vessel stabilization by promoting the interactions between endothelial cells, pericytes, and the extracellular matrix (Pryor et al., 2010; Yancopoulos et al., 2000). Mice that lack Ang1 have a less complex vascular network, as it is displayed by rounded endothelial cells that are poorly

associated with pericytes and the extracellular matrix (Suri et al., 1996). Ang1-null mice die at embryonic day 12.5 (Suri et al., 1996). In contrast, Ang2 binding to Tie2 does not induce phosphorylation, but instead blocks the binding of Ang1 (Yancopoulos et al., 2000). Studies have shown that Ang2 is an antagonist for Tie2 signaling, as transgenic overexpression of Ang2 disrupts capillary formation in the mouse embryo, exerting an effect very similar to that seen in Ang1-null mice (Maisonpierre et al., 1997). Ang2 is highly expressed during angiogenesis and its binding to Tie2 counteracts the effect of Ang1 and promotes destabilization and remodeling of the capillary (Hoier et al., 2011; Maisonpierre et al., 1997; Yancopoulos et al., 2000).

1.6.3 Modulation of Ang/Tie signaling during exercise

The concentration of the angiopoietins can fluctuate depending on whether the capillary network is in a latent or active state. Vessel stability is promoted when Ang1 level is greater than Ang2, and when the concentration of Ang2 outcompetes Ang1, vessel instability is promoted and new capillary growth occurs (Gale & Yancopoulos, 1999; Hoier et al., 2011; Lloyd et al., 2003). Lloyd and colleagues observed increases in Ang2-to-Ang1 ratio during exercise training, and Tie-2 mRNA expression was also upregulated and peaked at 8 days of treadmill running (Lloyd et al., 2003). The ratio between basal Ang2-to-Ang1 mRNA levels in human skeletal muscle was elevated after repeated bouts of single-legged exercise (Gustafsson et al., 2007). Ang1 gene expression was increased after 6 weeks of endurance training in humans, with no change in Ang2 levels (Timmons et al., 2005). Switching of the angiopoietin ratio can determine the regulatory response at different stages of angiogenesis. We hypothesize

the upregulation of Tie2 during exercise training is a marker of vascular stability and maturation of the capillary network.

1.6.4 Matrix metalloproteinases

The process of capillary growth requires the sprouting of new capillaries from pre-existing vessels. The expansion involves the progression of endothelial cell migrating into the interstitial matrix by degrading the basement membrane matrix (Kräling et al., 1999). Matrix metalloproteinases (MMPs) produced by endothelial cells are proteases that have the ability to cleave an assortment of extracellular matrix proteins. As reviewed by Haas, these proteins contribute to endothelial network formation which plays a major role in regulating both physiological and pathological angiogenesis (Haas, 2005). MMP-2 and MT1-MMP (MMP-14 or membrane type 1 MMP) are the most well described types that are associated with capillary growth. MMP-2 is secreted from endothelial cells and requires MT1-MMP on the cell surface to activate its proteolytic functions (Murphy et al., 1999). Although MT1-MMP is produced at very low levels in quiescent endothelial cells (Haas, 2005), stimulated muscles have been shown to increase the production of both MT1-MMP and MMP2 (Haas et al., 2000; Urso et al., 2009). Acute high-intensity resistance exercise also increase MMPs transiently in myofibres (Urso et al., 2009). The increase in MMP activity is said to be an early occurrence in active skeletal muscle which lead to the degradation of the basement membrane matrix, but it is not stimulated by increased in luminal flow (Brown & Hudlicka, 2003). MMP-2 protein levels have been shown to elevate as early as 12 and 24 hours after a single exercise bout (Olenich et al., 2013). When endothelial nitric

oxide production was blocked, an elevation of MMP-2 protein level was observed in rat extensor digitorum longus muscle (Milkiewicz et al., 2006b). The type of mechanical stimulus is one of the key determinants of MMP production in skeletal muscle. High-impact and high mechanical stress exercises that involve eccentric contraction seem to induce the greatest effect (Urso et al., 2009).

1.7 Angiostatic factors

A number of angiogenic inhibitors have been shown to be essential under physiological conditions in regulating and maintaining the vascular network in a quiescence state.

1.7.1 TSP-1

Thrombospondin-1 (TSP-1) is a large glycoprotein that mediates cell-to-cell and cell-to-matrix interactions (DiPietro et al., 1996). TSP-1 exerts its angiostatic functions by inhibiting endothelial cell proliferation (Iruela-Arispe et al., 1999), migration (Tolsma et al., 1993), and tube and lumen formation (Iruela-Arispe et al., 1991; Tolsma et al., 1997). Its effects are mediated by the TSP-1 domain, TSP-1 type-1 repeats (TSR1-3) (Bonnefoy et al., 2008; Olfert & Birot, 2011). The interaction of TSP-1 activation and TSR1-3 inhibits VEGF induced VEGFR2 activation by phosphorylation (Olfert & Birot, 2011). TSP-1 can also prevent VEGF release from the extracellular matrix by hindering MMP-9 activation (Rodriguez-Manzaneque et al., 2001). The deletion of TSP-1 in mice has led to an increase in skeletal muscle capillarization (Malek & Olfert, 2009). This indicates that TSP-1 has mechanistic control over vessel growth. Exercise detraining

induced an increase in TSP-1 protein expression, which coincided with skeletal muscle atrophy and capillary regression (Roudier et al., 2010). Acute, but not chronic, exercise increases TSP-1 mRNA expression, which indicates that the reduction in angiogenic inhibitors by training would allow angio-adaptation to occur (Hoier et al., 2011; Olfert et al., 2006; Slopack et al., manuscript in progress). The upregulation of TSP-1 seen with acute exercise and detraining may prevent unnecessary capillary growth to occur, and down regulation of TSP-1 during prolonged exercise may be important for angiogenesis to take place in skeletal muscle.

1.7.2 Endostatin

Endostatin is a 20 kDa protein fragment released from collagen XVIII that exerts its anti-angiogenic properties by inhibiting endothelial cell proliferation, migration, and tube formation (O'Reilly et al., 1997). This potent angiogenic inhibitor hinders with VEGF-induced VEGFR2 signaling (Y.-M. Kim et al., 2002), and also prevents the catalysis of MMP-2 and MT1-MMP by restricting the activation of proMMP-2 (Kim et al., 2000). Olenich and colleague have shown that endostatin protein levels in mouse skeletal muscle were elevated as early as 2 hours post-acute exercise (Olenich et al., 2013). A single bout of cycling exercise was also sufficient to increase the plasma level of endostatin in humans (Rullman et al., 2007). This process was reversed in human subjects that have undergone endurance training for 6 months (Brixius et al., 2008). The role of endostatin with exercise is not well understood. Future studies should examine whether endostatin is involved in maintaining vascular homeostasis.

1.7.3 Semaphorin

Semaphorin (Sema) constitutes a large family of transmembrane and secreted glycoproteins that are known to be involved in axonal guidance during neural development (Mark et al., 1997). The Sema family is categorized into eight classes. Class 1 and 2 are expressed in invertebrates, class 3-7 are found in vertebrates, and class 8 is specific to viruses (Goodman et al., 1999). All of the members have a conserved ~500 amino acid extracellular Sema domain (Kolodkin et al., 1993). Semaphorin signaling commences by binding to one of its class-specific receptors. Neuropilins (NRPs) are receptors for class 3 semaphorins (Sema3) (Kolodkin et al., 1997). NRPs are expressed in neurons, endothelial cells, and tumor cells (Gagnon et al., 2000; Soker et al., 1998). NRPs have been credited with facilitating capillary growth, as they also interact with VEGF (Klagsbrun et al., 2002; Soker et al., 1998; Soker et al., 2002). However, recent literature has determined that certain members of the Sema3 family affect the vasculature in an inhibitory manner through NRPs.

Sema3 consist of 6 secreted proteins, Sema3A through Sema3F (Goodman et al., 1999). Sema3F is expressed in endothelial cells (Guttmann-Raviv et al., 2007; Staton, 2011), and has been shown to inhibit tumor angiogenesis (Kessler et al., 2004). *In vitro* studies revealed that both Sema3A and Sema3F inhibit tube formation and migration of human dermal microvascular endothelial cells with and without the presence of VEGF (Staton, 2011). Guttmann-Raviv et al. showed that human embryonic kidney cells (HEK293) co-expressing Sema3A and Sema3F repelled endothelial cells, which caused areas of denuded zone in culture. Sema3F also induces endothelial apoptosis, and collapse the F-actin cytoskeleton (Bielenberg et al., 2008; Guttmann-

Raviv et al., 2007). Similar to VEGF-A, Sema3F is a ligand of NRP2 (Klagsbrun et al., 2002; Kolodkin et al., 1997). The overlapping binding site for NPR2 may suggest that functional competition between VEGF-A and Sema3F contributes to determining whether angiogenesis occurs (Geretti et al., 2007). The regulatory factors that govern the expression of Sema3F remain undetermined.

1.7.4 Vasohibin

Vasohibin (VASH-1) is expressed and secreted from the endothelium, and serves as a negative feedback regulator of angiogenesis (Watanabe et al., 2004). The p42 and p36 isoforms of VASH-1 are responsible for exerting anti-angiogenic activity (Olfert & Birot, 2011). This protein has been shown to inhibit endothelial cell proliferation, migration, and vascular tube formation in vivo and in vitro (Watanabe et al., 2004). Kishlyansky et al. have found that VASH-1 protein levels were higher in less vascularized muscles compared to oxidative muscles, hence, its expression is muscle type specific (Kishlyansky et al., 2010). Rats that have undergone a single bout of running exercise showed an increase in VASH-1 protein expression in the plantaris muscle, and this increase was abolished after 3-5 days of training (Kishlyansky et al., 2010). The similar expression patterns of VASH-1 and TSP-1 during exercise training suggests that the production of these two, and perhaps other, anti-angiogenic factors may be coordinated, so that they can complement one another in the inhibition of angiogenesis.

Synchronizing signals must be present in order to coordinate the balance between pro- and anti-angiogenic factors. The Forkhead Box “O” (FoxO) transcription

factors have been established in regulating the transcription of numerous genes involved in capillary growth and regression. Recent work of Roudier et al. has elucidated the role of FoxO proteins in regulating the expression of TSP-1 in ischemic skeletal muscle (Roudier et al., 2013). The next section will discuss the contribution of FoxO proteins in orchestrating the pattern of angiogenesis.

1.8 FoxO

The forkhead (Fox) family of transcription factors is classified by the presence of a 110 amino acid DNA binding domain (Kaufmann & Knöchel, 1996; Lai et al., 1993). It is comprised of over 80 members that have been identified in various species (Kaufmann & Knöchel, 1996). Members of the forkhead family have been shown to play vital roles during development and in the adults in regulating cellular differentiation and proliferation (Biggs et al., 2001). The FoxO subclass including FoxO1, FoxO3a, FoxO4, and FoxO6 are mammalian homologs (Anderson et al., 1998; Biggs et al., 2001; Furukawa-Hibi et al., 2002). FoxO1 and FoxO3a are highly expressed in endothelial cells (Biggs et al., 2001; Furuyama et al., 2000; Potente et al., 2005). While FoxO4 has relatively low expression in the endothelium, it has been shown to be expressed in the skeletal muscle (Biggs et al., 2001; T Furuyama et al., 2000; Potente et al., 2005). FoxO6 is strictly expressed in the brain (Jacobs et al., 2003). FoxO proteins exert their effects in the nucleus by binding to a forkhead responsive element on the promoters of downstream targets to mediate gene transcription, and initiate the coordination of proteins involved in the regulation of apoptosis and cell cycle transition (Brunet et al., 1999; Furuyama et al., 2000; Huang & Tindall, 2007). FoxO proteins are

phosphorylated by Akt on the serine or threonine residues which is located downstream of PI3K pathway (Brunet et al., 1999; Kops & Burgering, 2000). The activation of FoxO by Akt promotes 14-3-3 chaperone protein interaction that assists in the translocation of FoxO from the nucleus to the cytoplasm, where they may be targeted for proteasomal degradation (Brunet et al., 1999; Sunayama et al., 2005).

1.8.1 FoxO in vascular development

Mammalian FoxO proteins modulate a wide variety of cellular functions in cardiovascular tissues. Their role in the developing vasculature has been demonstrated by Hosaka et al. and Furuyama et al. in a transgenic model, where FoxO1-deficient mice displayed embryos and yolk sacs vessel impairment and died on embryonic day 11 (Furuyama et al., 2004; Hosaka et al., 2004). In contrast, FoxO3a- and FoxO4-null mice did not display embryonic abnormalities, and were similar to their wild type littermates (Furuyama et al., 2004; Hosaka et al., 2004). The unusual development of the vascular system in FoxO1-deficient mice was due to the insufficient response of endothelial cells to exogenous VEGF, which therefore disrupted an essential signaling pathway that is vital to normal vascular development (Furuyama et al., 2004). Furthermore, the development of endothelial specific FoxO1-deficient mice also displayed similar cardiovascular defects and embryonic lethality, thus leading to the conclusion that this effect is endothelial FoxO1 dependent (Sengupta et al., 2012). These studies strongly support an essential role of endothelial FoxO1 in the formation of new vasculature.

1.8.2 FoxO in adult angiogenesis

The role of FoxO appears to be reversed during postnatal development and adulthood. Contrary to FoxO deletion during mouse embryogenesis, FoxO1/3a/4-null induced at 4-5 weeks of age developed systemic hemangiomas (excessive growth of endothelial cells) that resulted in premature death (Paik et al., 2007). *In vitro* findings demonstrated enhanced proliferation and survival in endothelial cells derived from FoxO1/3a/4 deleted mice, and this effect was caused by the downregulation of Sprouty2 (Paik et al., 2007). Robust endothelial cell proliferation and migration were observed in *ex vivo* 3D muscle explant culture excised from mice with conditional endothelial cell-directed deletion of FoxO1/3a/4 (Roudier et al., 2013). Moreover, silencing of endogenous FoxO1 or FoxO3a gene expression with small interfering RNA led to a profound increase in endothelial cell migratory responses and tube formation *in vitro* (Potente et al., 2005). Thus, FoxO subclass 1 and 3a exhibit angiostatic potential, which indicates their crucial role in the regulation of endothelial cell homeostasis.

1.8.3 FoxO and physical activity

FoxO has recently been shown to regulate the balance between pro- and anti-angiogenic factors in response to physical activity. The frequency of exercise performed influences the expression of FoxO. An acute exercise bout induced increase in FoxO1 and FoxO3a protein level, while repeated exercise of 14 days significantly downregulated FoxO1 and FoxO3a protein expression (Slopack et al., manuscript in progress). Previous work in our lab showed that FoxO transcription factors are able to negatively regulate the process of capillary growth, as mice with endothelial cell directed

deletion of FoxO1/3/4 (FoxO∆) have accelerated angiogenesis in response to repeated bouts of endurance exercise (Slopack et al., manuscript in progress). Furthermore, the expression patterns of TSP-1 during exercise training are similar to FoxO1 and FoxO3a, and the increase in TSP-1 with acute exercise is abolished in FoxO∆ animals (Slopack et al., 2013, manuscript in progress). This suggests that FoxO regulates downstream angiostatic targets such as, TSP-1 during exercise (Roudier et al., 2013; Slopack et al., manuscript in progress).

The regulation of FoxO during physical activity could be a consequence of increased activation of Akt, which occurs in response to fluid shear stress (Dimmeler et al., 1998; Milkiewicz et al., 2011). Increased VEGF also could lead to the phosphorylation of FoxO1 and FoxO3a via the activation of Akt (Potente et al., 2005). Both shear stress and VEGF expression can be elevated with short term exercise (Breen et al., 1996; Gustafsson et al., 2002; Hudlicka et al., 2006), and return to basal level with prolonged training (Hudlicka et al., 2006; Lloyd et al., 2003; Olfert & Birot, 2011; Pryor et al., 2010). The exercise response elicits the angio-adaption that occurs, and this process may be mediated by factors such as FoxO.

1.9 Summary

The coordinated regulation of angiogenic and angiostatic factors can control the behaviour of capillaries in response to different environmental stimuli, such as the frequency of exercise training. These regulatory signals orchestrate the extent to which capillarization occurs. As recently demonstrated by our lab, an increase in capillary-to-fibre ratio was observed at 7 days of treadmill running in FoxO∆ mice (compared to 14

days in wildtype mice) (Slopack et al., manuscript in progress). Beyond its role in controlling TSP1 production, the accelerated vessel growth suggests that FoxO transcription factors may also be able to negatively regulate genes involved in sprouting, which promotes enhanced sprouting in their absence.

Tip cell formation in other models is strictly guided by Dll4/Notch signaling, while subsequent maintenance and maturation of the vascular network is regulated by Dll1 and Tie2. It is probable that these factors are involved in sprouting and maturation of the capillary network that is known to occur in skeletal muscle in response to exercise training. These molecules have not been investigated within the skeletal muscle microcirculation, and nothing is known about the expression pattern of these factors in skeletal muscle in response to exercise.

1.9.1 *Study objectives*

Angiogenic growth factors that determine endothelial sprout and maturation must be coordinated in order to bring forth their intended effects. Transcription factors can act as those coordinating signals to ensure the occurrence of effective cellular responses. I hypothesize that FoxO transcription factors regulate genes such as Dll1, Dll4, and Tie2, which control the processes of sprout formation and capillary maturation in skeletal muscle. I focused on 2 major objectives to address this hypothesis.

Objectives:

1) To investigate the expression pattern of Dll1, Dll4, and Tie2 in skeletal muscle during endothelial sprout formation and capillary maturation.

2) To determine whether FoxO1 and FoxO3a regulate the expression of angiostatic factors (vasohibin, semaphorin 3F) and tip cell sprouting and maturation related proteins Dll1, Dll4, and Tie2 during endurance exercise.

Chapter 2: Methods

Ethical Approval

Animal studies were approved by York University Committee on Animal Care, and performed in accordance with Animal Care Procedures at York University and the American Physiological Society's Guiding principles in the Care and Use of Animals.

Mouse Model of Exercise Training

In the short term training protocol, twenty female FVB/n mice, age 9 weeks, were purchased from Charles River (Saint-Constant, QC, Canada). They were housed on a 12:12 light-dark cycle with water and food ad libitum. 3 days prior to the exercise training regimen, mice were placed on the treadmill (#91447-3) for 15 minutes at a speed of 15m/min for acclimatization. The mice were divided at random in to the sedentary group (n=5), 1 day of training (n=4), 1 day of training plus 2 hours of recovery (n=4), 3 day of training (n=4), and 5 days of training (n=4). Treadmill running was performed at a speed of 25m/min for 60 minutes for their respective training bouts. The sedentary group was placed on the treadmill daily to control for handling and environment. Mice were fasted 4 hours prior to their final bout of exercise, and they were anaesthetized (isoflurane/oxygen inhalation) immediately following their last bout of training, 2 hours after an acute bout of exercise, or rest. Gastrocnemius, plantaris, and soleus muscles were excised, weighed, and frozen in liquid nitrogen. The mice were euthanized by exsanguination.

Mouse Model of FoxO deletion

MxCre+:FoxO1,3,4$^{L/L}$ (FoxO$^{\Delta}$) and MxCre-: FoxO1,3,4 (FoxO $^{L/L}$) mice on a FVB/n background were bred at York University. They were housed on a 12:12 light-dark cycle with water and food ad libitum. The FoxO1, FoxO3a, and FoxO4 genes of these mice have one exon that is flanked by two LoxP sites. FoxO$^{\Delta}$ mice also contained the Cre- recombinase (Cre) trans-gene under the control of Mx1-promoter. Mice were given 3 intraperitoneal injections every other day of 400ug of Polyinosinic-polycytidylic acid (Poly I:C) (#tlrl-picw Invitrogen) (2mg/mL) at approximately 4 weeks of age. Poly I:C induces the production of interferon-α/β (IF-α/β) by binding to the toll-like receptor 3. The Mx-promoter is then activated by IF-α/β to initiate Cre transcription (Kühn et al., 1995). In FoxO$^{\Delta}$ animals, activating the expression of Cre recombinase will remove the portion of the FoxO genes flanked by the two LoxP sites, resulting in a near complete deletion of FoxO within endothelial cells (Paik et al., 2007) and other cells that express toll-like receptor 3. Poly I:C injection does not modify FoxO expression in FoxO $^{L/L}$ as they lack the Cre trans-gene.

Extended training

In the extended training protocol, FoxO$^{\Delta}$ (n=42, 21 female, 21 male) or FoxO $^{L/L}$ (n=42, 21 female, 21 male) were divided two weeks after Poly I:C injection. Each group were further allocated into their exercise groups: sedentary (n=6), 1 day of training (n=6), 7 days of training (n=6), 14 days of training (n=6), 28 days of training (n=6) and 28 days sedentary (n=6). Acclimatization to the treadmill (15 minutes at a speed of 15m/min) was performed 5 days prior to the training regimen. Running exercise was

performed at a speed of 25 m/min, 60 minutes per day. The 28 day trained mice ran on the treadmill 5 days/week for 4 weeks. The sedentary group was placed on the treadmill daily to control for handling and environment. Following the final bout of exercise, the mice were given a 2 hour recovery period before the administration of anaesthesia (isoflurane/oxygen inhalation). Gastrocnemius, soleus, plantaris, tibialis anterior, extensor digitorum longus, and heart were excised, weighed, and frozen in liquid nitrogen. The mice were euthanized by exsanguination. The plantaris was removed, weighed, embedded in cryogel, and then frozen in liquid nitrogen and cooled isopentane for histochemistry.

The above 3 animal studies were conducted earlier for the thesis of Dara Slopack. I have contributed to the exercise training, muscle excision, and further analyses as described below.

RNA extraction from muscle:

~10mg of gastrocnemius muscle was used for RNA extraction via RNeasy Fibrous Tissue Mini Kit (Qiagen) as per manufacturer's instructions. The extracted RNA was stored at -20 °C.

qRT-PCR

qRT-PCR was performed on extracts from the gastrocnemius muscle of the sedentary, 1 day, 7 day, 14 day, and 28 day training groups. RNA was reverse transcribed to cDNA using Cells-to cDNA ^{TM} kit (Ambion) according to the

manufacturer's instructions (9ul of RNA per sample). cDNA was diluted in 60uL of RNase free water. 4ul of cDNA was combined with TaqMan® universal Fast PCR master mix (Applied Biosystems) and specific Taqman probes and primers (Applied Biosystems), as listed: Dll1 (#Mm01279269_m1), Dll4 (#Mm01338015_m1), Tie2 (#Mm0043243_m1), Vasohibin (#Mm00616592_m1), Sema3F (#Mm00441325_m1), and HPRT-1 housekeeping gene, as a control (#Mm00446968_M1). Samples were assessed in duplicate. qRT-PCR was performed in the following thermal conditions: 50 °C for 30 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute [7500 fast RT-PCR system (Applied Biosystems)]. To determine the amount of target sample mRNA, the average cycle threshold (C_T) was calculated and compared to the average cycle threshold of HPRT-1 for the same samples with the formula $\Delta C_T = \text{Average}C_{T\,(\text{sample})} - \text{Average}C_{T\,(\text{control})}$. The ΔC_T of the training samples was then compared to the ΔC_T of the sedentary samples by computing $\Delta\Delta C_{T,}$ where $\Delta\Delta C_T = \Delta C_{T(\text{training})} - \Delta C_{T(\text{sedentary})}$. The amount of target amplification relative to the experimental control was calculated by the formula $2^{-\Delta\Delta CT}$.

3D muscle explant angiogenesis assay

All tools and solutions were sterilized before surgery. Soleus muscles were carefully isolated from FVB/n mice. Each soleus was divided into 4–6 fragments or biopsy samples of approximately 3 X 3 mm in size. Biopsy samples were rinsed in cold sterile PBS and kept on ice until the next step. Then samples were rinsed 3 times with 5% FCS in Dulbecco's modified Eagle medium (DMEM; Gibco) and then embedded within a type 1 collagen gel as described below. The collagen gel was prepared using

acid-soluble type 1 collagen from calf skin (5 mg/ml in 0.1% acetic acid; Elastin Products Co.). For 1 ml of mix, 120 ul of type 1 collagen (5 mg/ml), 400 ul of 2.5X DMEM, 50 ul of 0.1 N NaOH, and 10 ul of penicillin/streptomycin (Gibco) were mixed together. The solution was kept on ice (to avoid unwanted polymerization) until aliquoted into precooled 24-well tissue culture plates (250 ul of mix per well). Soleus biopsy samples were incubated for 1–2 min in cold collagen mix and then were placed at the center of the well. After polymerization (30 min at 37°C in CO2 incubator), DMEM containing 5% serum was added to each well. Medium was changed every other day.

Muscle biopsy samples were cultivated in medium with 5% FBS for different durations to allow the analysis of the successive steps of endothelial cell proliferation/migration into the collagen matrix until their assembly into primitive vascular tubes. After 4 d of culture in our conditions, endothelial cells were still in a dynamic phase of migration. Endothelial cells were stained for alkaline phosphatase activity by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (FAST BCIP/NBT; Sigma-Aldrich) for 45 min at 37°C. Pictures were acquired using an Axio Imager (Zeiss) equipped with an AxioCam camera (Zeiss).

Immunofluorescence staining of explants

Muscle explants were stained with NG2 (#AB5320 Millipore), then counterstained with Isolectin (#FL-1101 Vector) and DAPI (#P5521 Promega) (1:1500 in PBS), then mounted onto slides with Immunofluor mounting medium and viewed with a Zeiss Axiovert 200M light microscope. Explants were viewed using 10X or 40X objectives,

and images were captured at using Lambda 10-2 camera, and MetaMorph Imaging software.

Muscle whole mount staining

Plantaris muscle cross-sections and longitudinal sections were cryosectioned to ~10μm and ~15 μm thickness, respectively, and then mounted on microscope slides. The sections were fixed with 3.7% paraformaldehyde, and then blocked with 5% goat serum or 5% donkey serum diluted in phosphate-buffered saline. Immunostaining was performed with Dll1 (#H-265 Santa Cruz Biotechnology), Dll4 (#Ab7280 Abcam), Tie2 (#AF762 R&D Systems), or Pdgfb (#Ab23914 Abcam) antibodies. The staining was visualized by the incubation with Alexa Fluor ® 488 goat anti-rabbit (#A11008 Invitrogen), Alexa Fluor ® 568 donkey anti-goat (#A11057 Invitrogen), or DyLight 549 goat anti-rabbit (#DI-1549 Vector Laboratory) secondary antibodies. The muscle sections also were counterstained with isolectin (#FL-1101 Vector) to detect capillary endothelial cells or α smooth muscle actin (#C6198 Sigma-Aldrich). Negative controls were first incubated with serum respected to the host of the secondary antibody, and at the same concentration as the primary antibody of the gene of interest. The immunostained muscle sections were viewed by Zeiss Observer Z1 confocal microscope with 488nm and 555nm lasers using 10x, 20x, and 63x objectives. Images were captured using Zen 2010 software set to line 4 averaging, and emission signals at 75% for FITC and 63% for rhodamine. Z step sizes were taken at ~4.5 μm/step at 10x, ~1.5μm/step at 20x, and ~0.83μm/step at 63x. At each magnification, image gain and offset were adjusted between the different targets and magnification to optimize the

quality of the signal, and these settings were kept the same between different exercise time points and for negative control of the same target.

Statistical analysis

Statistical analyses were performed using Student's t test, 1-way or 2-way ANOVA as appropriate, using Prism 4 (GraphPad Software Inc.). For 1-way and 2-way ANOVA analyses Bonferroni post-hoc test was used. The results were considered to be statistically significant at values of $p < 0.05$.

Chapter 3: Results

Dll4/Notch signaling promotes endothelial tip cell formation in mouse retina and zebrafish embyro (Hellström et al., 2007; Jakobsson et al., 2010), and subsequent vascular maintenance is thought to be regulated by Dll1 and Tie2 pathways (Limbourg et al., 2007; Yancopoulos et al., 2000). To determine whether these molecules may play a role in angiogenic sprouting in skeletal muscle, I examined their transcript levels as well as their protein localization within the vasculature of skeletal muscle under resting and exercise conditions. Using this model, we have found that exercise training induces the formation of new capillaries within 14 days (Slopack et al., manuscript in progress).

Short term exercise influences Dll1, Dll4, and Tie2 expression

Transcript levels were assessed in mouse gastrocnemius muscle after acute and short term aerobic exercise. Dll1 mRNA level was significantly increased immediately after a single bout of exercise, returned to basal level after 2h of recovery, then increased again immediately after 3d of exercise ($p<0.001$, n=6) (Figure 3-1A). Dll4 expression was not influenced by a single exercise bout, but showed a tendency for reduction at 5d of exercise ($p=0.06$, n=6) (Figure 3-1B). Tie2 was significantly down-regulated at 5d of exercise compared to sedentary ($p<0.05$, n=6) (Figure 3-1C).

Utilization of 3-D explant to examine sprout morphology

The muscle explant model was used initially as a tool to assess the localization of tip cell proteins. I selected this method because endothelial cells migrate into the 3-D

collagen matrix from the soleus muscle biopsy 4 days after embedment, forming many filopodia-like structures. Thus, immunofluorescence staining of the muscle explants would allow localization of tip cell proteins to the extending endothelial cell sprouts. To validate the model, the muscle biopsy was stained with isolectin to identify vascular endothelial cells, NG2 (a pericyte marker), and DAPI to locate nuclei. NG2 positive stain overlapped the isolectin stain. Pericytes appeared to surround the migrating endothelial cells, even at the furthest point of the cell migration. Images produced from the 3-D muscle explant did not provide good quality staining that could display EC sprout morphology, and presumably the localization of tip cell targets (Figure 3-2). Consequently, I did not pursue this model further, and chose to investigate the expression of putative tip cell proteins in histological sections of mouse muscle.

Dll4 expression is decreased with exercise training

Immunostaining for Dll4 was performed on skeletal muscle from sedentary, 7 day and 14 day trained mice. Both cross-sections and longitudinal sections of the muscle were assessed, to allow for a lateral view of the capillaries relative to muscle fibres and a side view that displays the length of the capillaries, respectively. The two viewing angles allowed us to better locate the expression of the protein of interest within the vascular network. As negative control, immunostaining using only species specific serum respected to the appropriate secondary showed no positive staining within the muscle or vasculature (Figure 3-13 – Figure 3-16). The Dll4 signals overlapped isolectin stain, which suggests that Dll4 is widely expressed in capillaries and large vessels of mouse skeletal muscle. Both muscle cross-sections and longitudinal-sections displayed

a decrease in Dll4 staining intensity after 7d and 14d of treadmill running compared to sedentary mice (Figure 3-3, Figure 3-4).

Dll1 is expressed in sedentary and 7d of training, but reduced after 14d of training

To determine if Dll1 protein is expressed in skeletal muscle and altered during exercise, Dll1 expression was assessed by immunohistochemistry. Dll1 signal is strong in sedentary and 7d of training, but diminished at 14d of training (Figure 3-5, Figure 3-6). Positive Dll1 signals predominately overlapped isolectin staining, which suggests that Dll1 is expressed in capillaries and large vessels of mouse skeletal muscle. Dll1 expression also was found in lateral segments between adjacent capillaries in the longitudinal muscle sections from exercised mice (see arrowheads in Figure 3-6), this occurrence was most prominent at the 7d time point. The expression of Dll1 in this location may suggest the development of lateral branches, as the selection of tip cell is commencing.

Tie2 expression is present, but weak in sedentary and trained muscles

Tie2 expression in the endothelium has been shown to be associated with the recruitment of pericytes and smooth muscle cells (Patan, 1998; Peters et al., 2004) and thus may be an indicator of maturation and stabilization of the vascular network. Tie2 immunostaining was detectable within capillaries, but the staining was uniformly weak in muscles from both sedentary and trained mice (Figure 3-7, Figure 3-8).

Pdgfb is not a definitive marker of tip cell in skeletal muscle

Tip cells are frequently identified by the presence of Pdgfb in mouse retina (Hellström et al., 2007). To determine if Pdgfb is a marker of tip cells in skeletal muscle, the localization of Pdgfb was assessed by immunohistochemistry. Pdgfb was expressed in the skeletal muscle endothelium, but its expression was not restricted to tip cells (Figure 3-9 and Figure 3-10). Pdgfb staining also was observed in areas close to, but not overlapped with, isolectin positive capillaries in both cross-sections and longitudinal sections.

Dll1 and Pdgfb are expressed in endothelial cells, but not mural cells of skeletal muscle

Endothelial cells are closely associated with mural cells (pericytes or smooth muscle), and it is difficult to differentiate between the two at the light microscope level. To provide further evidence that Dll1 and Pdgfb are expressed in endothelial cells, and not in mural cells, longitudinal muscle sections from 7 days of training were stained with secondary antibody conjugated with Alexa Fluor 488 specific to the host of Dll1 and Pdgfb antibodies, then counterstained with α smooth muscle actin, a marker of smooth muscle cells and pericytes. Both Dll1 (Figure 3-11) and Pdgfb (Figure 3-12) signals were detected side-by-side to α smooth muscle actin positive staining, but the signals did not overlap.

Exercise training elicited temperal changes in FoxO1 and FoxO3a expression

Recent work from our lab had found that FoxO1 and FoxO3a protein expression were decreased after repeated bouts of exercise compared to a single bout (Slopack et

al., manuscript in progress). To examine FoxO1 and FoxO3a transcript levels during exercise training, we assessed gastrocnemius muscle from mice that remained sedentary, or underwent treadmill running for 1, 7, or 14 days. FoxO1 mRNA was significantly elevated after a single bout of exercise ($p<0.05$, n=6) (Figure 3-17A). This increase in FoxO1 mRNA was abolished after 14 days of training ($p<0.05$, n=6) (Figure 3-17A). FoxO3a mRNA was unchanged at day 1 compared to sedentary (n=6), but it significantly decreased after 7 and 14 days of training compared to a single exercise bout ($p<0.05$, n=6) (Figure 3-17B).

Dll1, Dll4, and Tie2 expression were influenced by FoxO during exercise training

In addition to observing the expression pattern, we were interested to know how these factors are regulated. Our lab recently demonstrated that FoxO transcription factors are able to negatively regulate angiogenesis, as mice with endothelial cell directed deletion of FoxO1/3/4 ($FoxO^{\Delta}$) have augmented endothelial cell proliferation and migration, and also display accelerated vessel growth in response to repeated bouts of endurance exercise (Slopack et al., manuscript in progress). To determine if FoxO regulates the expression of tip cell markers in muscle during exercise, Dll1, Dll4, and Tie2 mRNA levels were assessed in $FoxO^{L/L}$ and $FoxO^{\Delta}$ mice that remained sedentary, or underwent exercise training for 1, 7 or 14 days. Dll1 expression was significantly down-regulated in $FoxO^{L/L}$ mouse gastrocnemius muscle after 7 and 14 days of treadmill running, coinciding with the period of capillary sprouting ($p<0.001$, n=6) (Figure 3-18A). In $FoxO^{\Delta}$ mice, basal Dll1 mRNA was reduced compared to wildtype, and there was no further decrease with training ($p<0.05$, n=6) (Figure 3-18A). There

was no change in Dll4 mRNA with exercise (n=6) (Figure 3-18B). In FoxO$^{\Delta}$ mice, Dll4 expression was decreased at 7d and 14d compared to FoxO $^{L/L}$ sedentary (p<0.05, n=6) (Figure 3-18B). Tie2 expression was bi-phasic, increasing two-fold in animals trained for 1 and 14 days compared to sedentary counterparts (p<0.05, n=6) (Figure 3-18C). The up-regulation of Tie2 seen after 14 days of exercise was abolished in FoxO$^{\Delta}$ animals (p<0.01, n=6) (Figure 3-18C).

Influence of FoxO expression on angiostatic factors during exercise training

The process of endothelial sprout formation is also affected by the production of angiostatic factors. We previously found that the anti-angiogenic factor thrombospondin1 is regulated by FoxO proteins, leading us to hypothesize that FoxO proteins may coordinate the expression of multiple anti-angiogenic factors within skeletal muscle. Vasohibin expression was shown to be repressed in response to exercise training (Kishlyansky et al., 2010). Vasohibin mRNA levels were assessed to determine whether its expression in skeletal muscle is controlled by FoxO during exercise. Neither a single bout nor long-term exercise influenced vasohibin mRNA levels in FoxO$^{L/L}$ animals (n=5 for 1d, n=6 for Sed and 14d) (Figure 3-19). In FoxO$^{\Delta}$ mice, vasohibin mRNA expression was significantly up-regulated after a single bout of exercise compared to time matched FoxO$^{L/L}$ (p<0.05, n=5) (Figure 3-19).

Sema3F is reported to inhibit angiogenic sprouting, but its expression within skeletal muscle has not been reported to date. Sema3F expression was up-regulated after a single bout of exercise and returned to basal levels after 7d and 14d of training,

coinciding closely with the mRNA expression pattern of FoxO1 (p<0.05, n=6) (Figure 3-20A). Sema3F mRNA levels did not change with extended training (p=0.055, n=5 for Sed, n=6 for 28d) (Figure 3-20B). The increase in Sema3F mRNA expression seen at 1d was abolished in FoxO$^{\Delta}$ mice (p<0.05, n=5) (Figure 3-21).

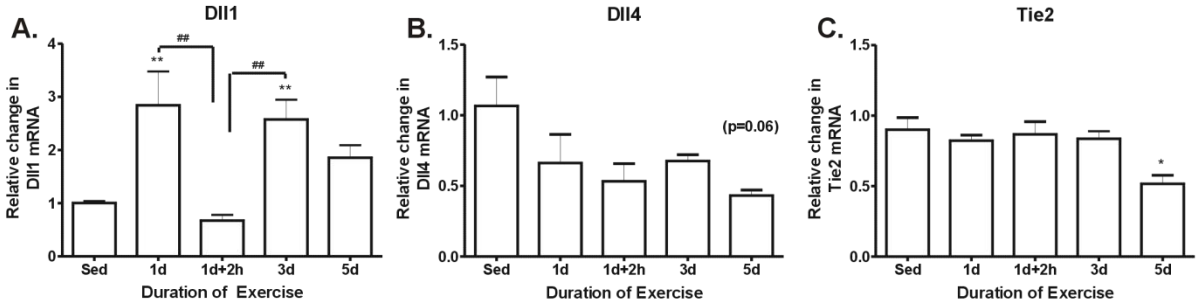


Figure 3-1. Short-term training mRNA analysis of Dll1, Dll4, and Tie2 expression.
Muscles were collected immediately after exercise from FVB/n mice trained for 1, 3, 5 days (1d, 3d, 5d), or remained sedentary (Sed). Muscles were collected 2 hours after an acute bout of exercise (1d+2h). A) Dll1 gene expression. (B) Dll4 gene expression. (C) Tie2 gene expression. Changes in Dll1, Dll4, and Tie2 mRNA expression were assessed by real time PCR. *=p<0.05 vs. Sedentary, **=p<0.001 vs. Sedentary, ##=p<0.001 vs. 1d+2h, 1-way ANOVA. (n=6/ group).

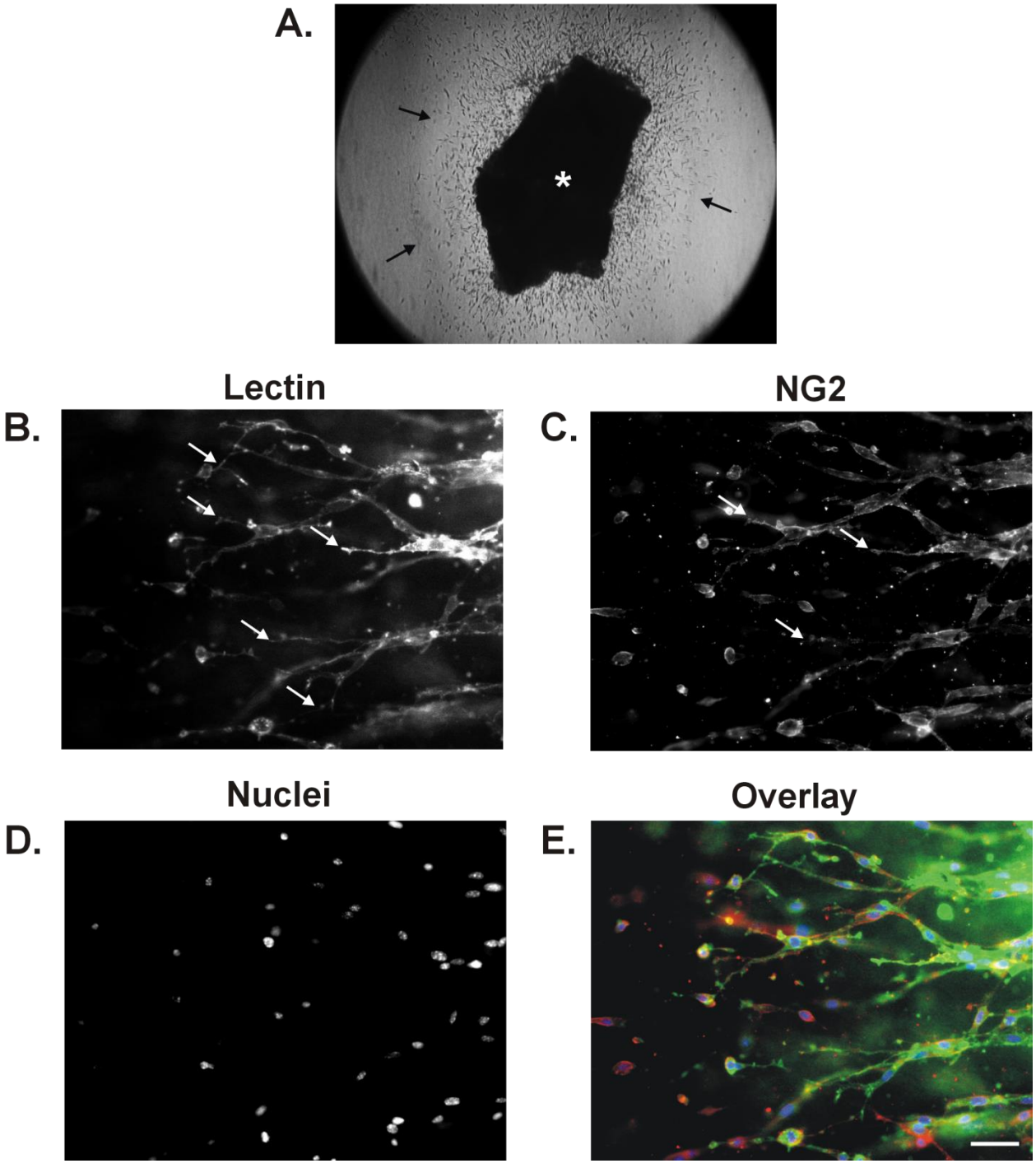

A.
*
Lectin
B.
NG2
C.
Nuclei
D.
Overlay
E.

Figure 3-2. Utilization of 3-D explant to examine sprout morphology and localization of tip cell targets in skeletal muscle.
A mouse soleus muscle biopsy was embedded within a 3-D collagen gel, and images were taken after 4 days of culture. (A) Phase image displaying alkaline phosphatase-stained cells (black arrows) migrating away from the muscle biopsy (asterisk). (B) Isolectin staining of vascular EC was used to identify cells that displayed tip-like characteristics (white arrows). (C) NG2 positive pericytes (white arrows). (D) Nuclear staining with DAPI. (E) Overlay of the three colour channels. (Bar = 50μm).

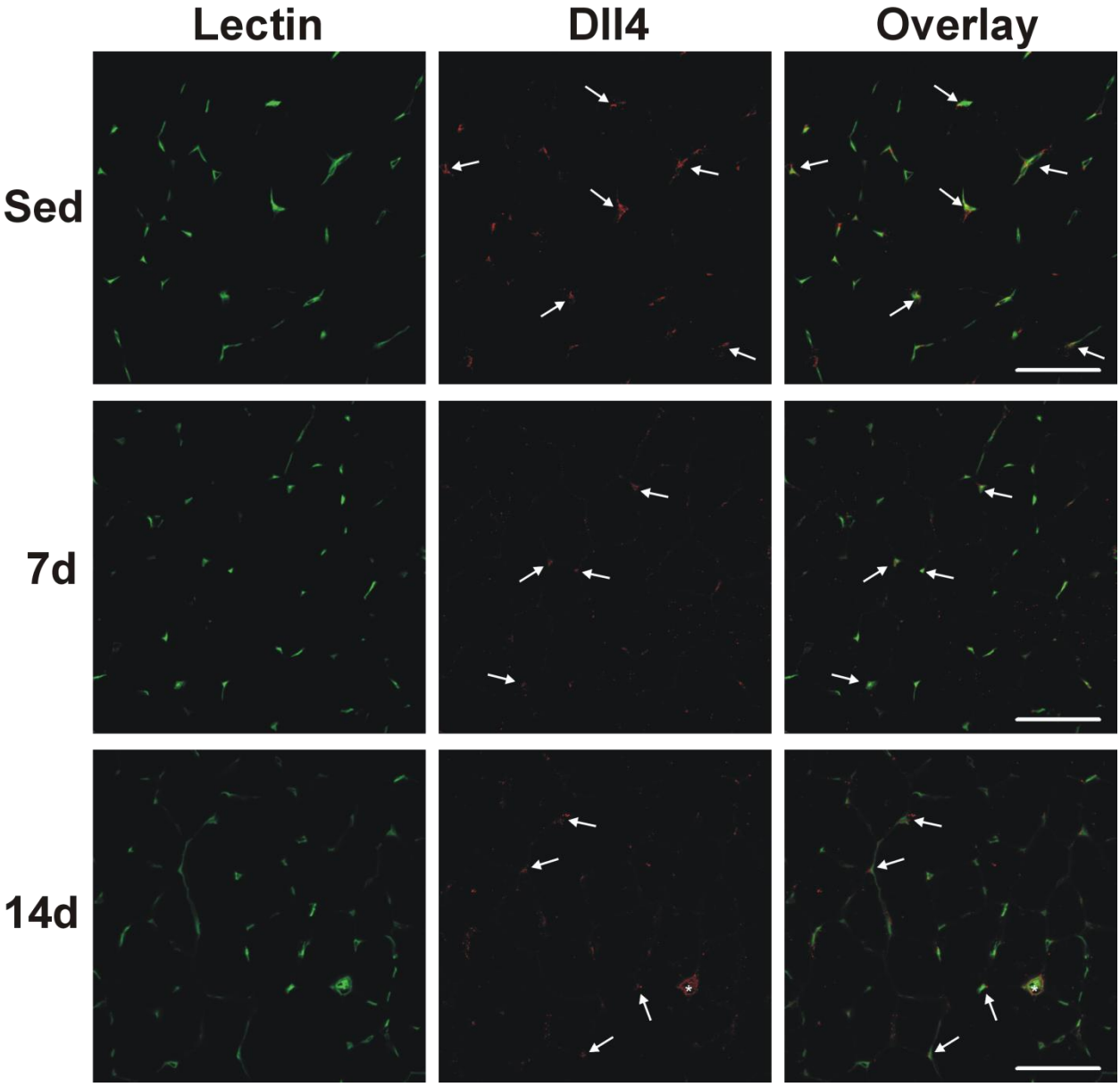
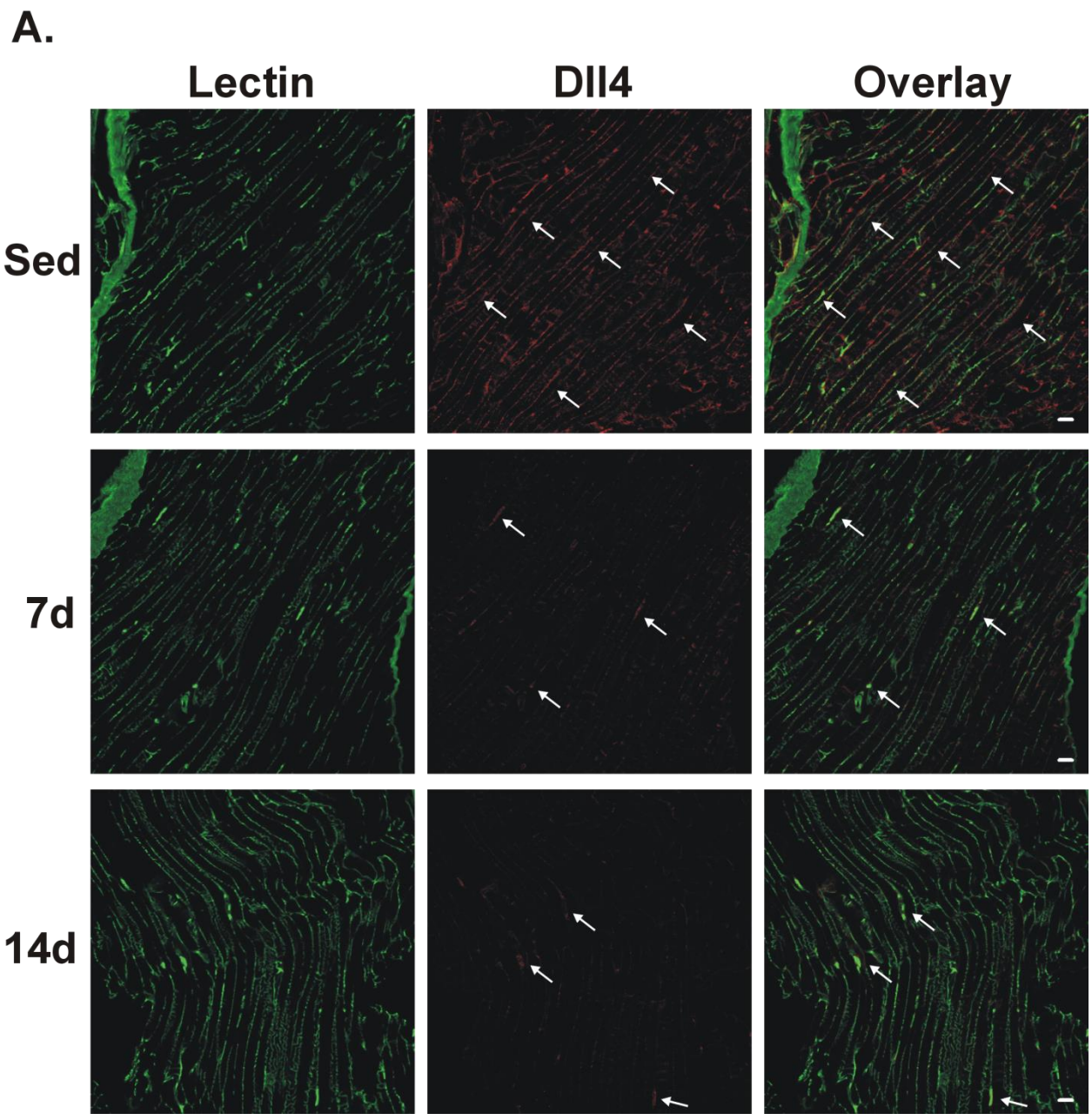
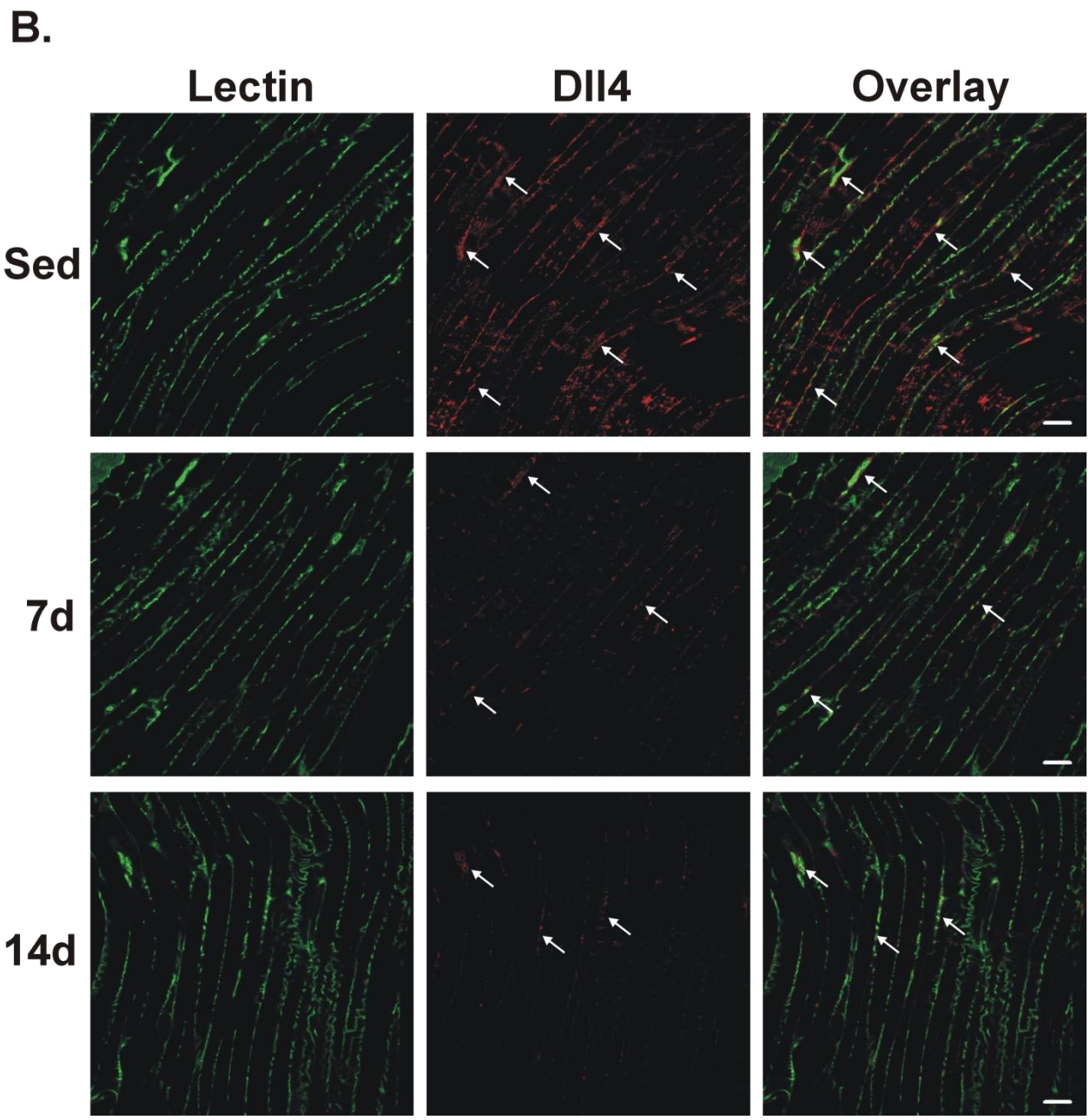


Figure 3-3. Decrease in Dll4 expression with exercise training in muscle cross-sections. Muscle cross-sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Dll4 (red) and isolectin for vascular EC (green). Images were viewed with 63x objective. Dll4 positive staining that overlaps isolectin (arrow). Dll4 positive staining that overlaps large vessels (asterisk). (Bar = 50μm).


A.
Lectin
Dll4
Overlay
Sed
7d
14d


B.
Lectin
Dll4
Overlay
Sed
7d
14d

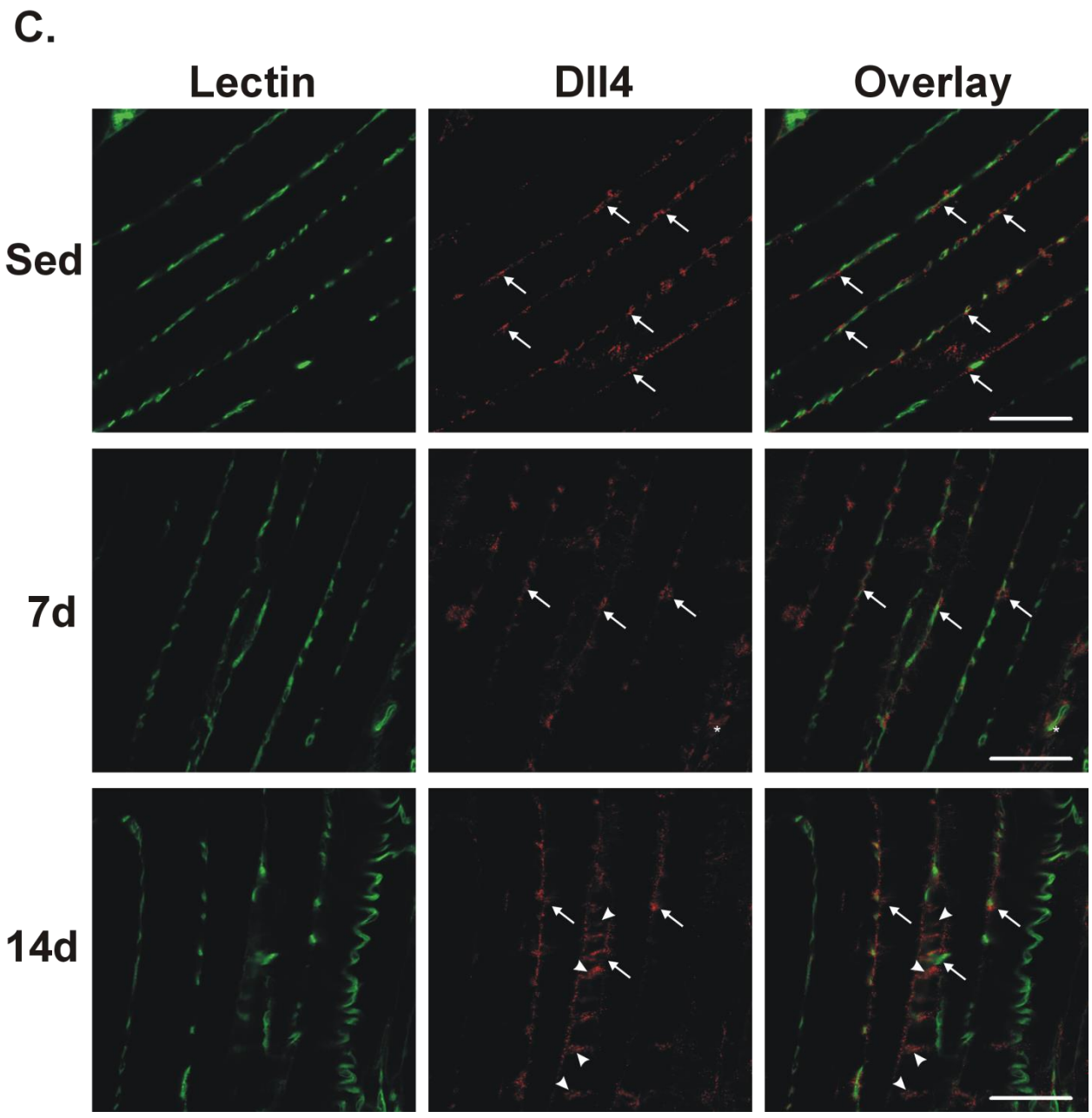

C.
Lectin
Dll4
Overlay
Sed
7d
14d

Figure 3-4. Decrease in Dll4 expression with exercise training in longitudinal muscle sections.
Longitudinal sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Dll4 (red), and isolectin for vascular EC (green). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. Dll4 positive staining was observed to overlap with isolectin (arrow) and could also be observed in large vessels (asterisk). Dll4 staining that does not overlap isolectin (arrowhead). (Bar = 50μm).

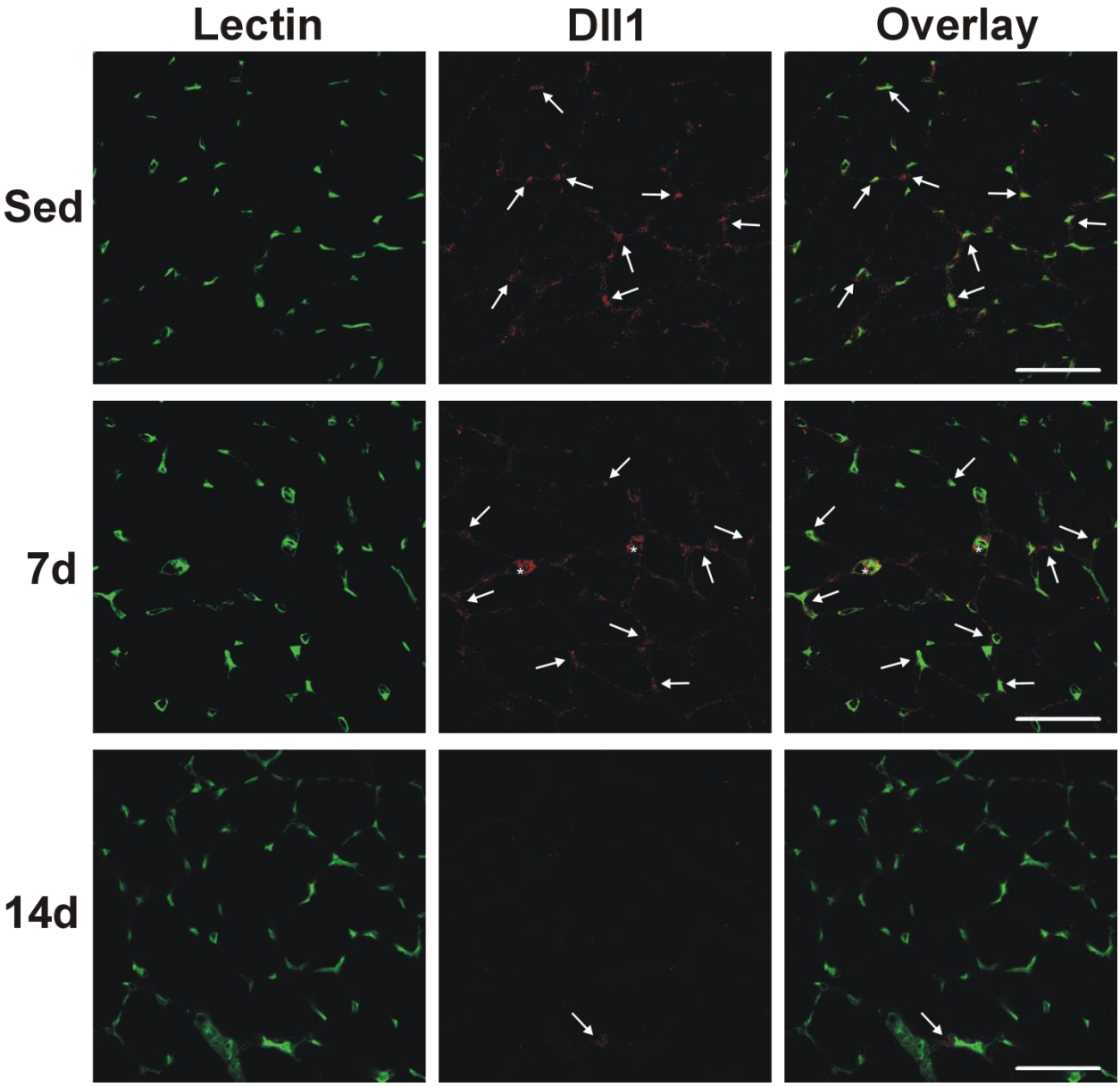
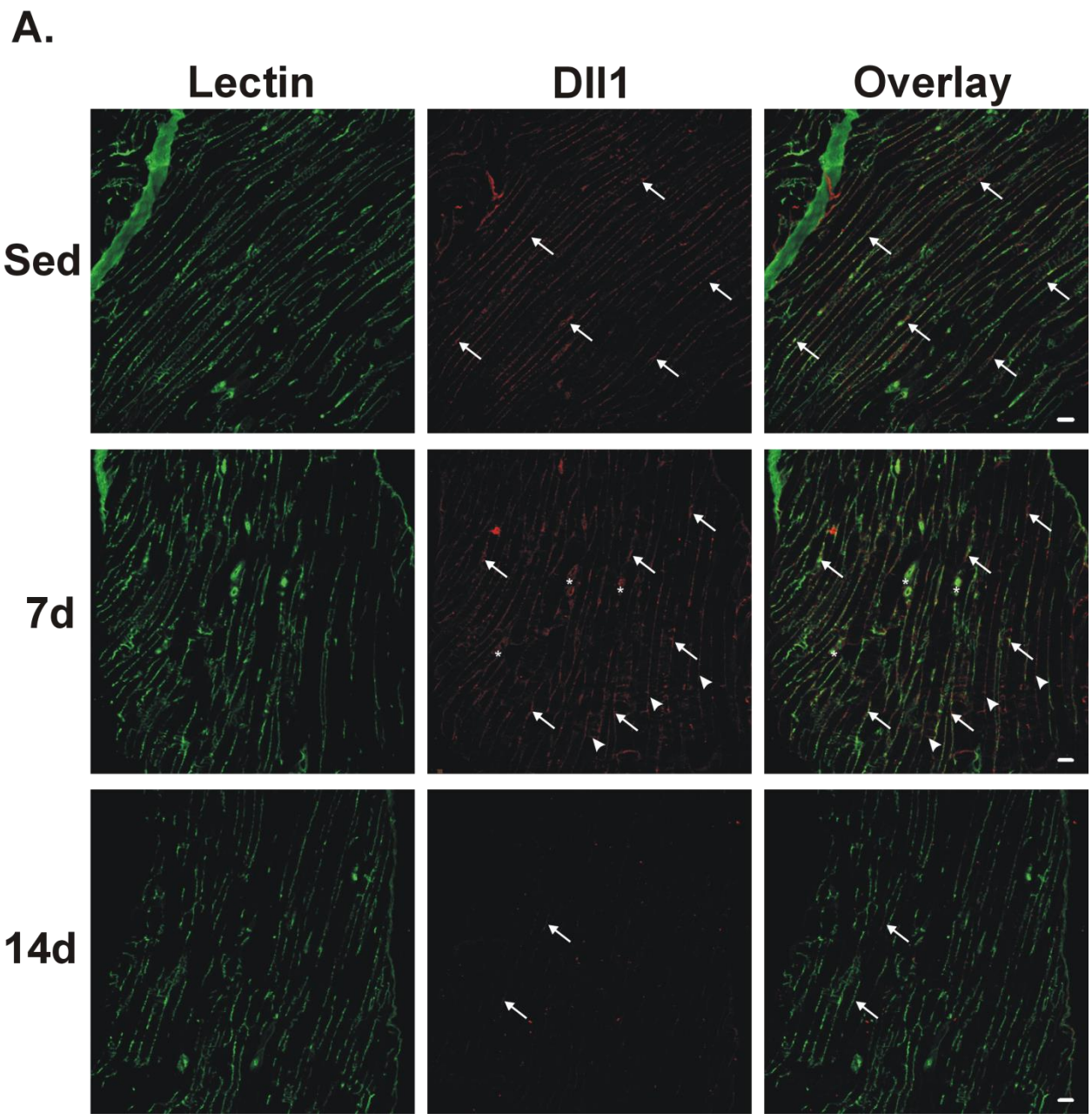
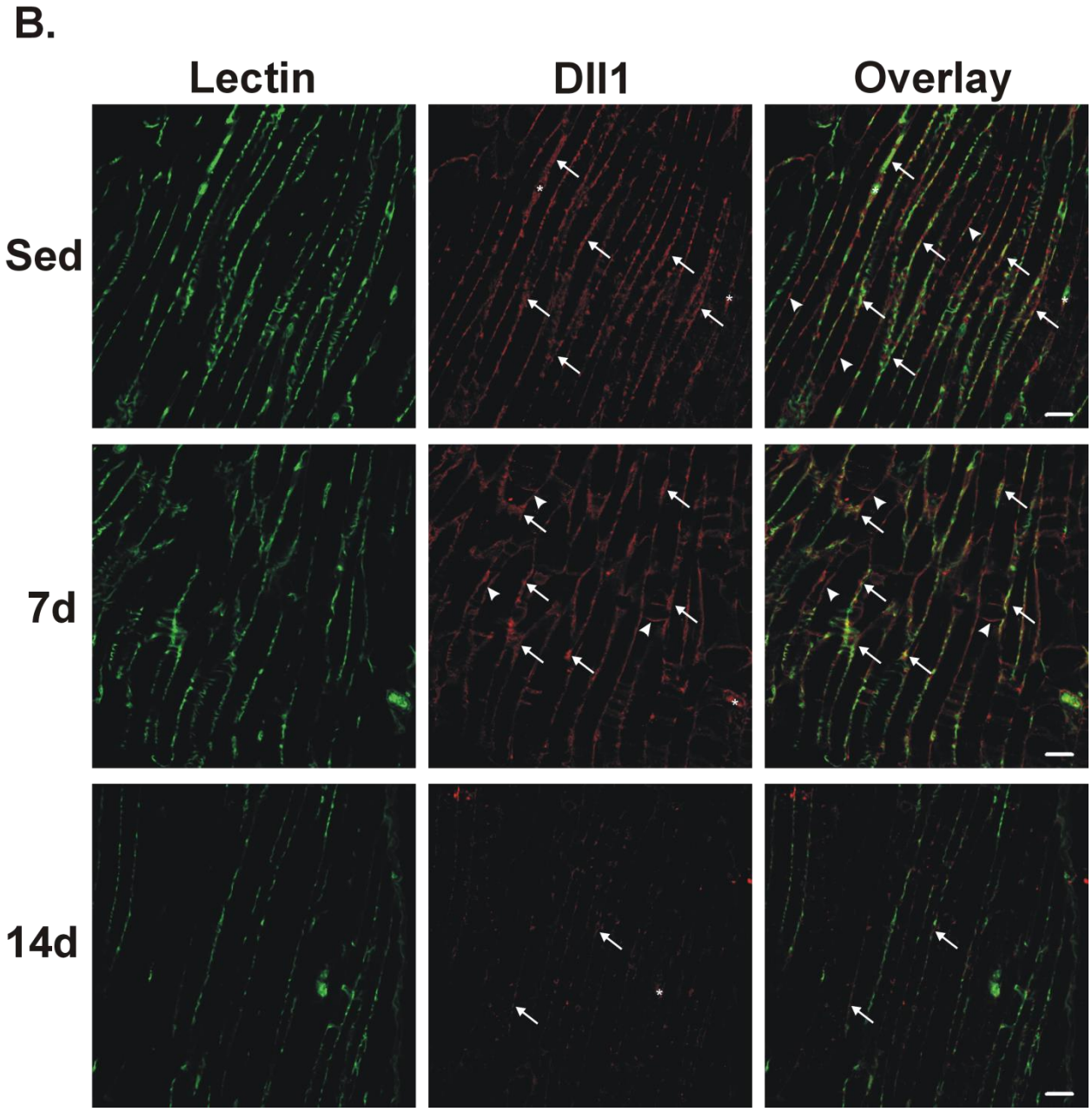


Figure 3-5. Dll1 is expressed in cross-sections of sedentary and 7d trained, but not in 14d trained muscles.
Muscle cross-sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Dll1 (red) and isolectin for vascular EC (green). Images were viewed with 63x objective. Dll1 positive staining was observed to overlap with isolectin (arrow) and could also be observed in large vessels (asterisk). (Bar = 50μm).


A.
Lectin
Dll1
Overlay
Sed
7d
14d


B.
Lectin
Dll1
Overlay
Sed
7d
14d

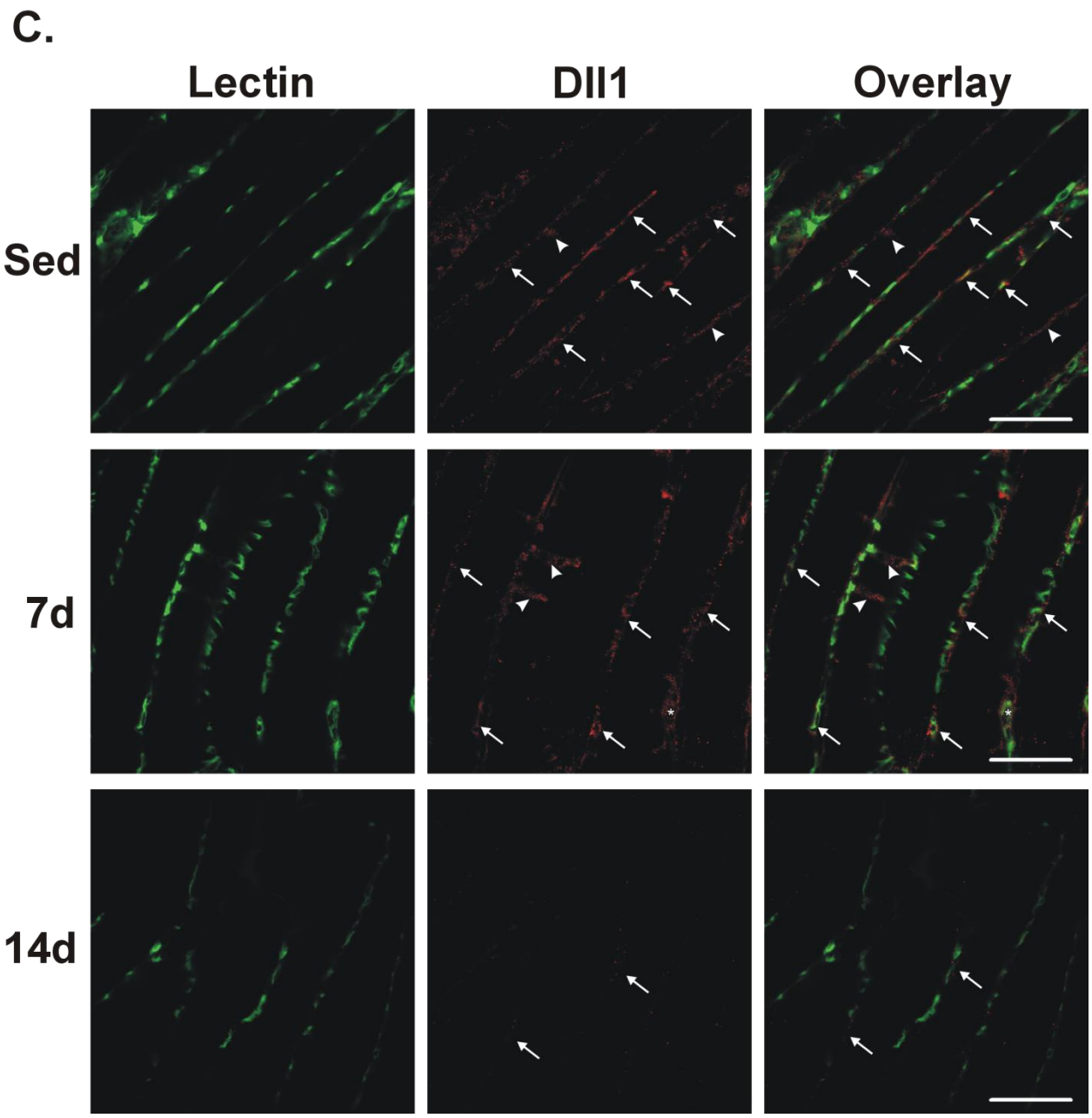

C.
Lectin
Dll1
Overlay
Sed
7d
14d

Figure 3-6. Dll1 is expressed in longitudinal sections of sedentary and 7d trained, but not in 14d trained muscles.
Longitudinal sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Dll1 (red), and isolectin for vascular EC (green). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. Dll1 positive staining was observed to overlap with isolectin (arrow) and could also be observed in large vessels (asterisk). Dll1 staining that does not overlap isolectin (arrowhead). (Bar = 50μm).

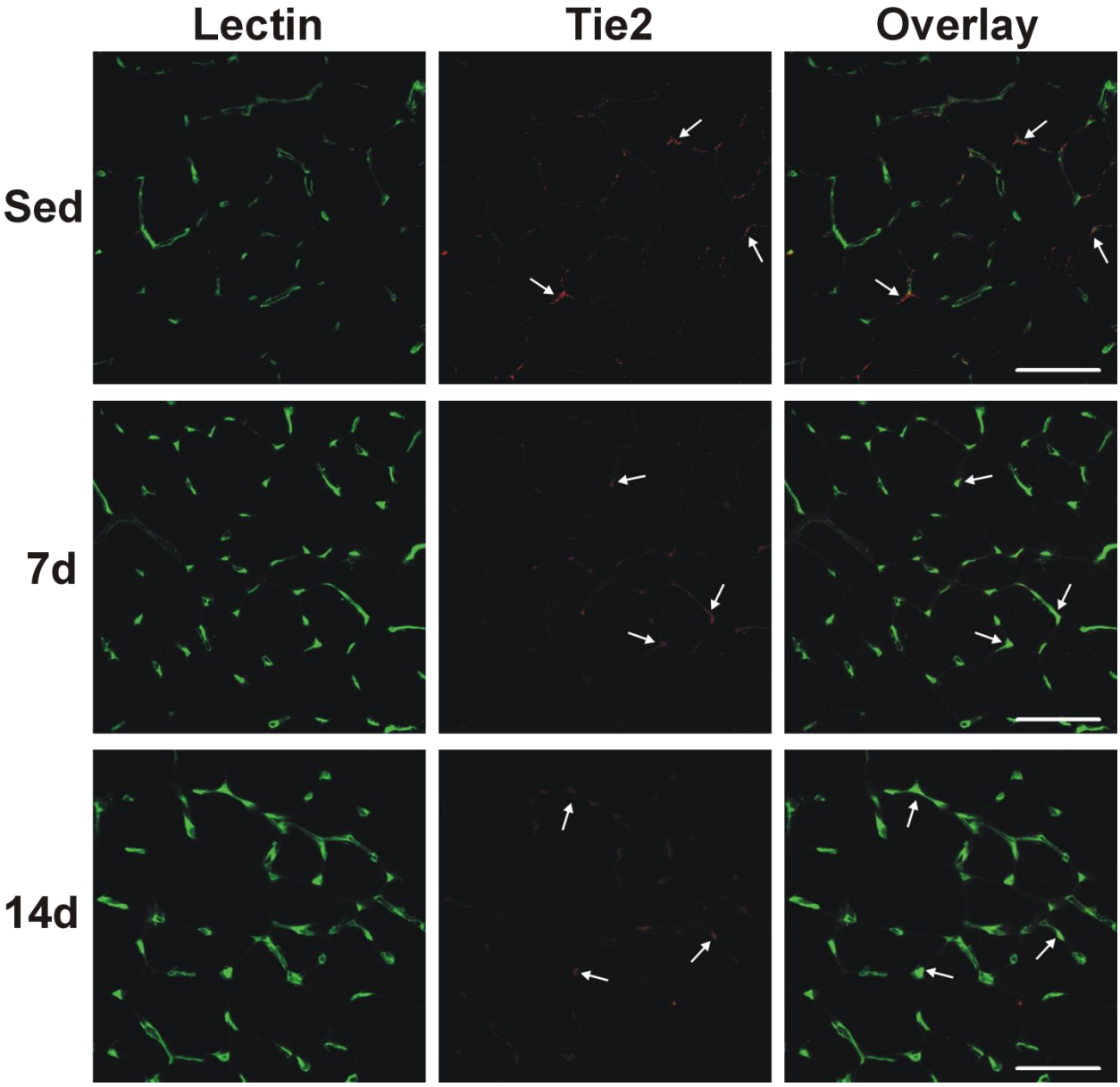
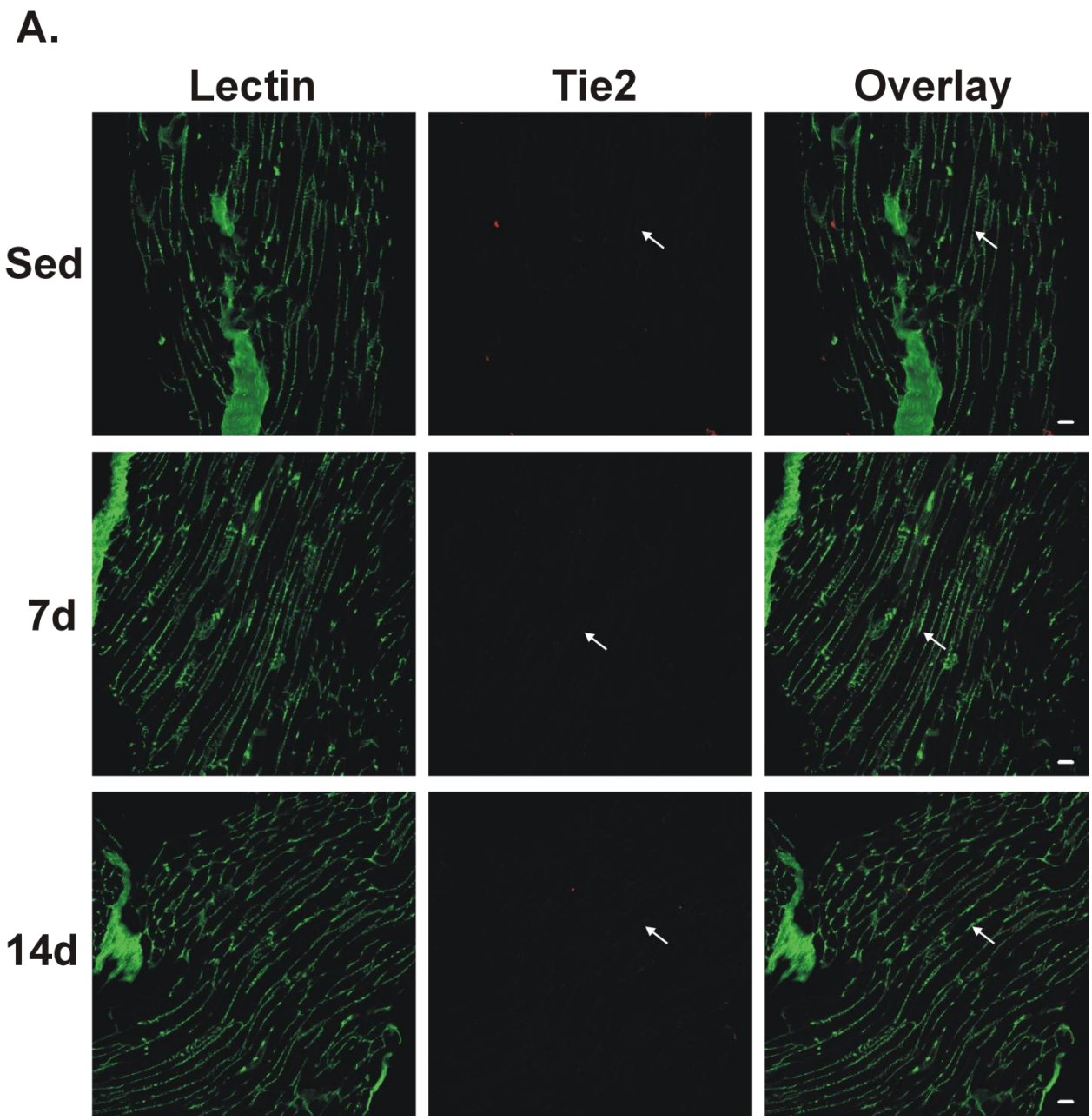
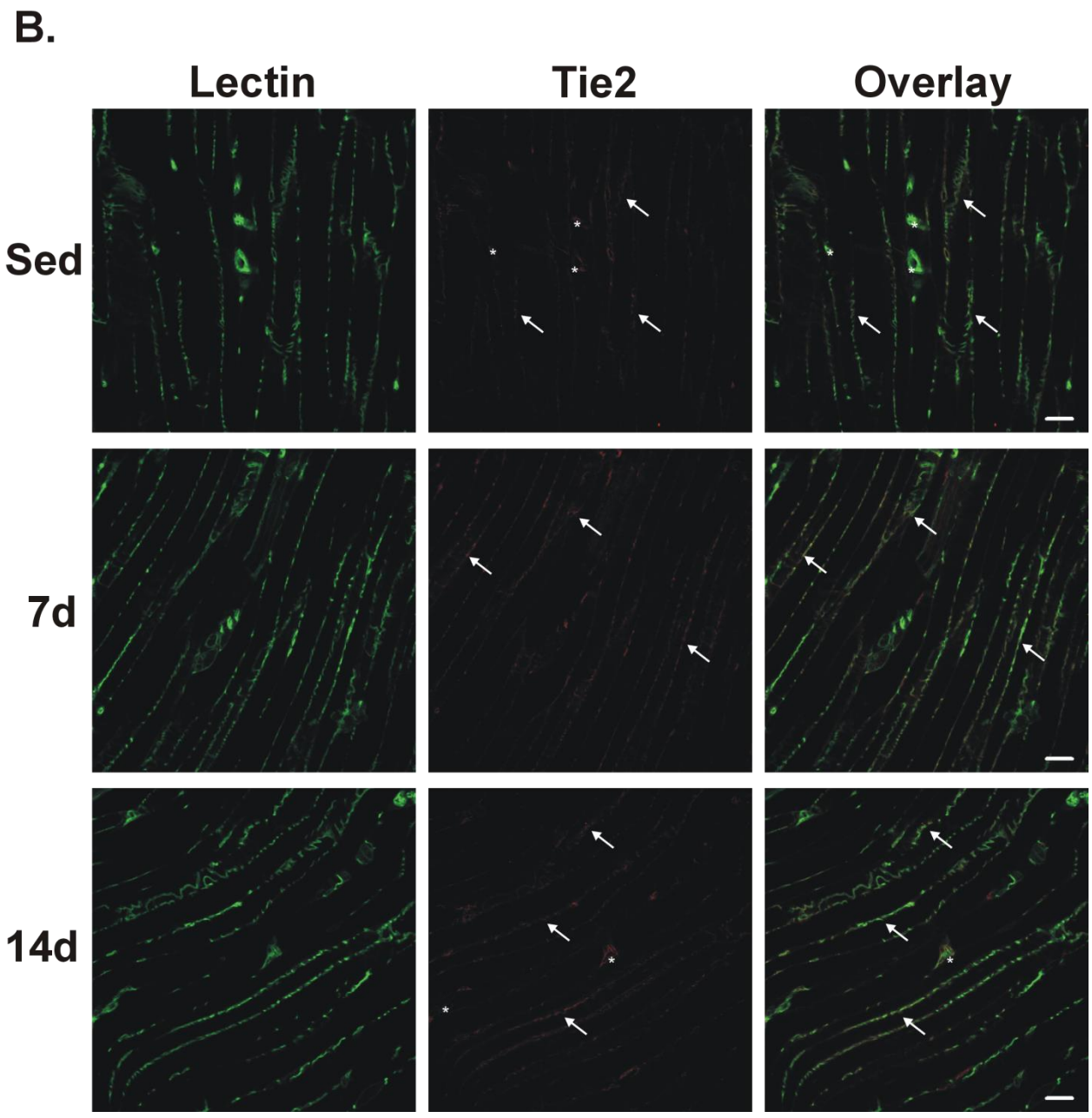


Figure 3-7. Tie2 expression in cross-sections is present, but weak in sedentary and trained muscles.
Muscle cross-sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Tie2 (red), and isolectin for vascular EC (green). Images were viewed with 63x objective. Tie2 positive staining was observed to overlap with isolectin (arrow). (Bar = 50μm).


A.
Lectin
Tie2
Overlay
Sed
7d
14d


B.
Lectin
Tie2
Overlay
Sed
7d
14d

C.

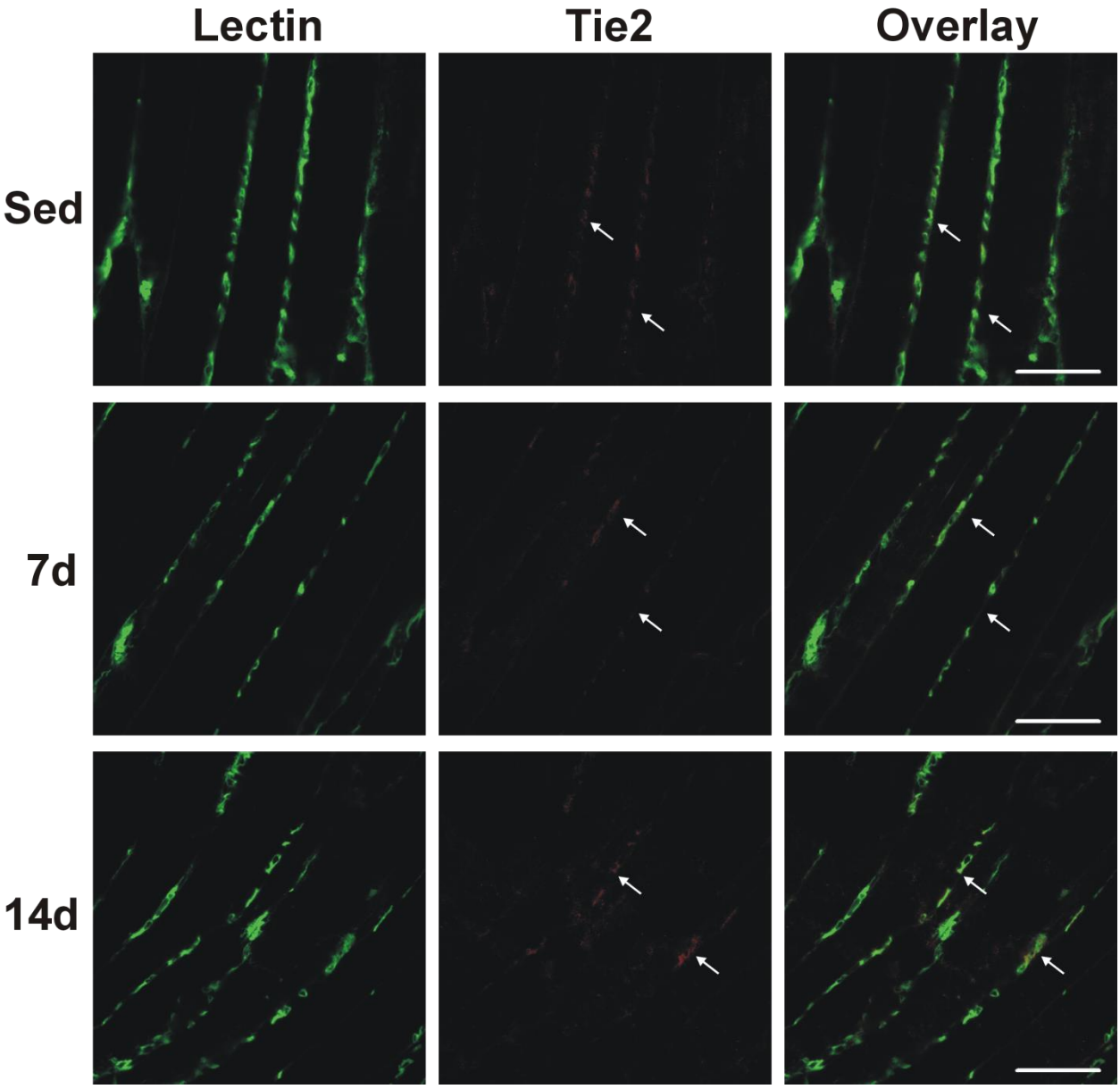


Figure 3-8. Tie2 expression in longitudinal sections is present, but weak in sedentary and trained muscle.
Longitudinal sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Tie2 (red), and isolectin for vascular EC (green). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. Tie2 positive staining was observed to overlap with isolectin (arrow) and could also be observed in large vessels (asterisk). (Bar = 50μm).

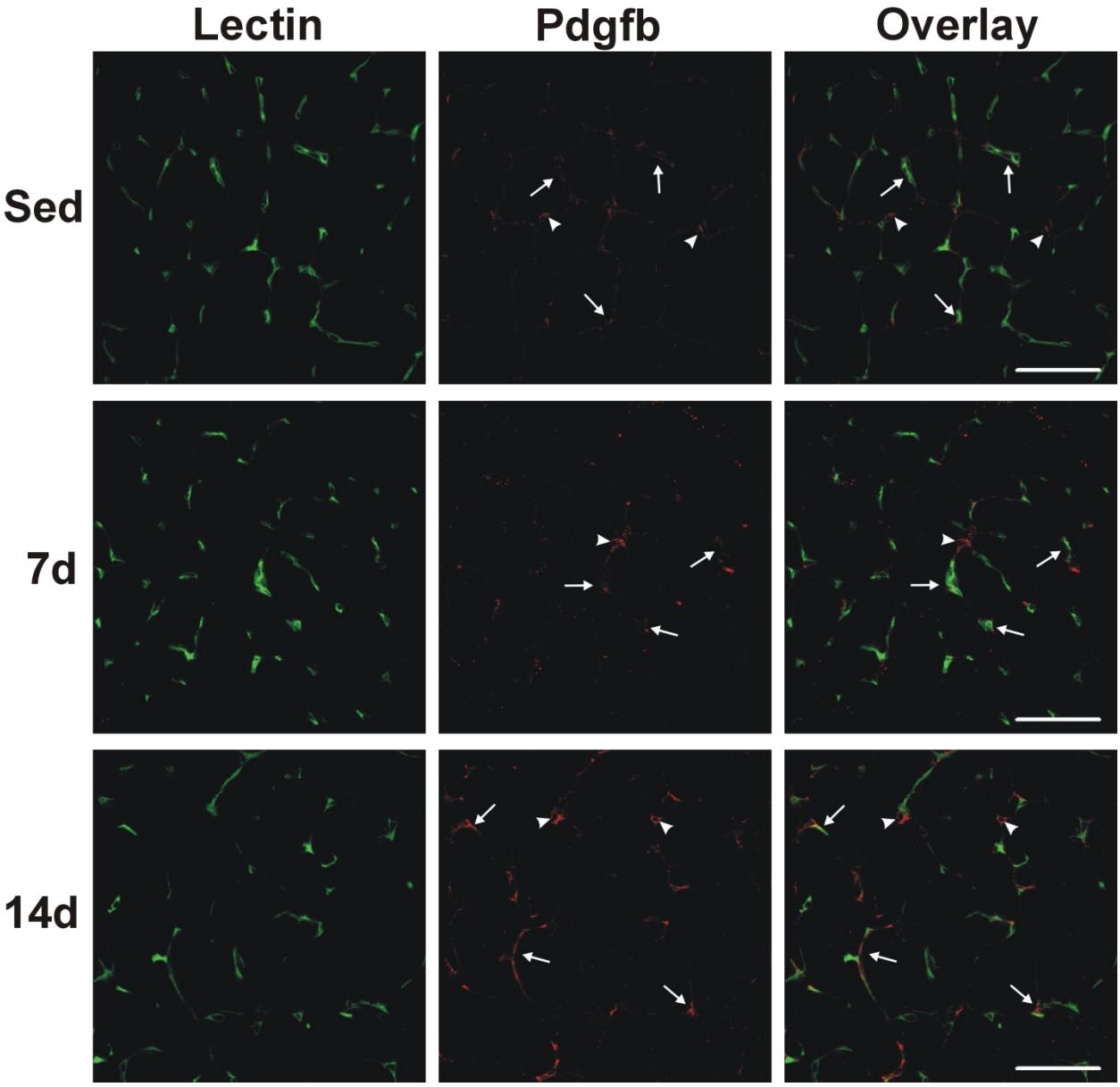
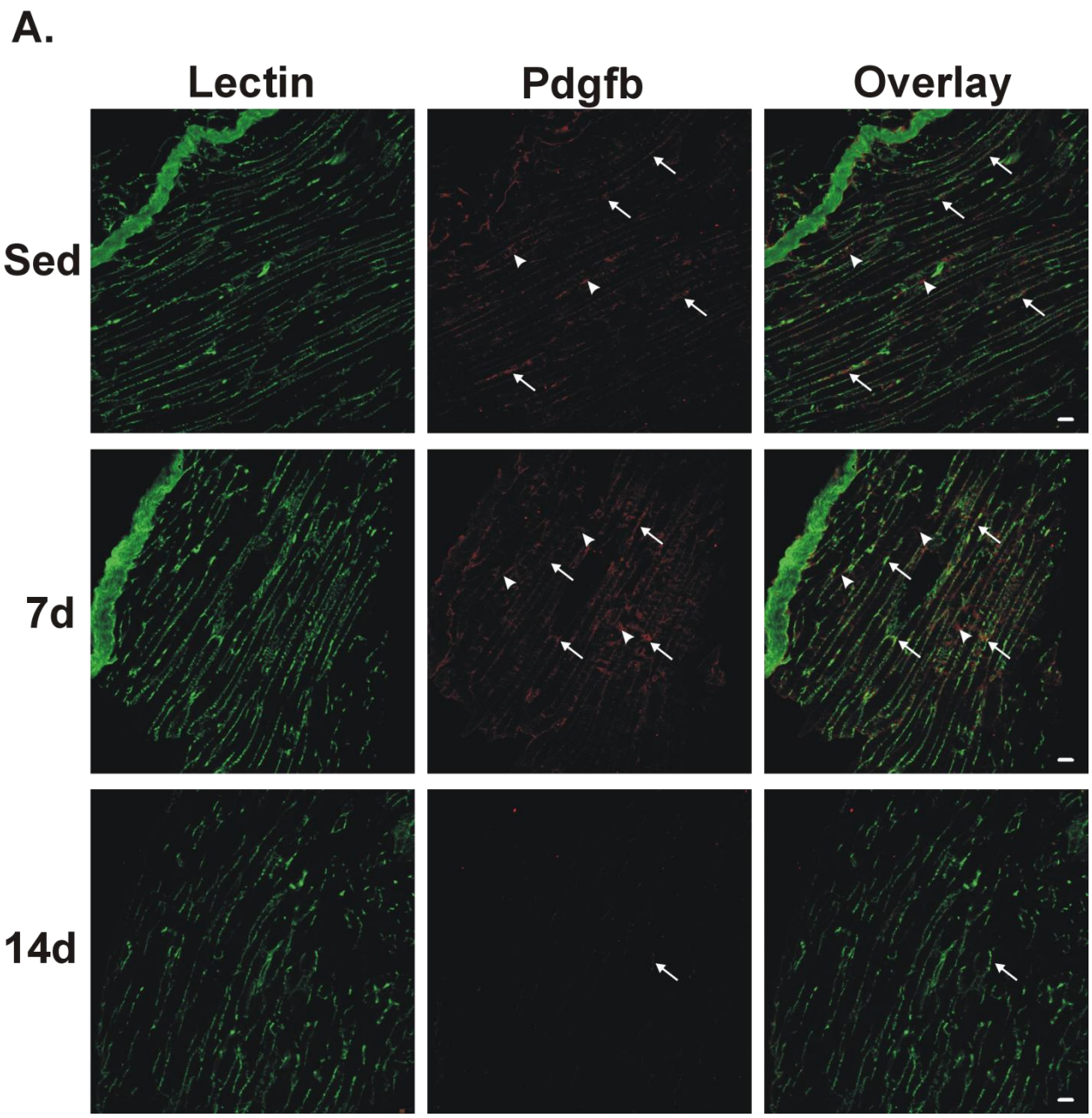
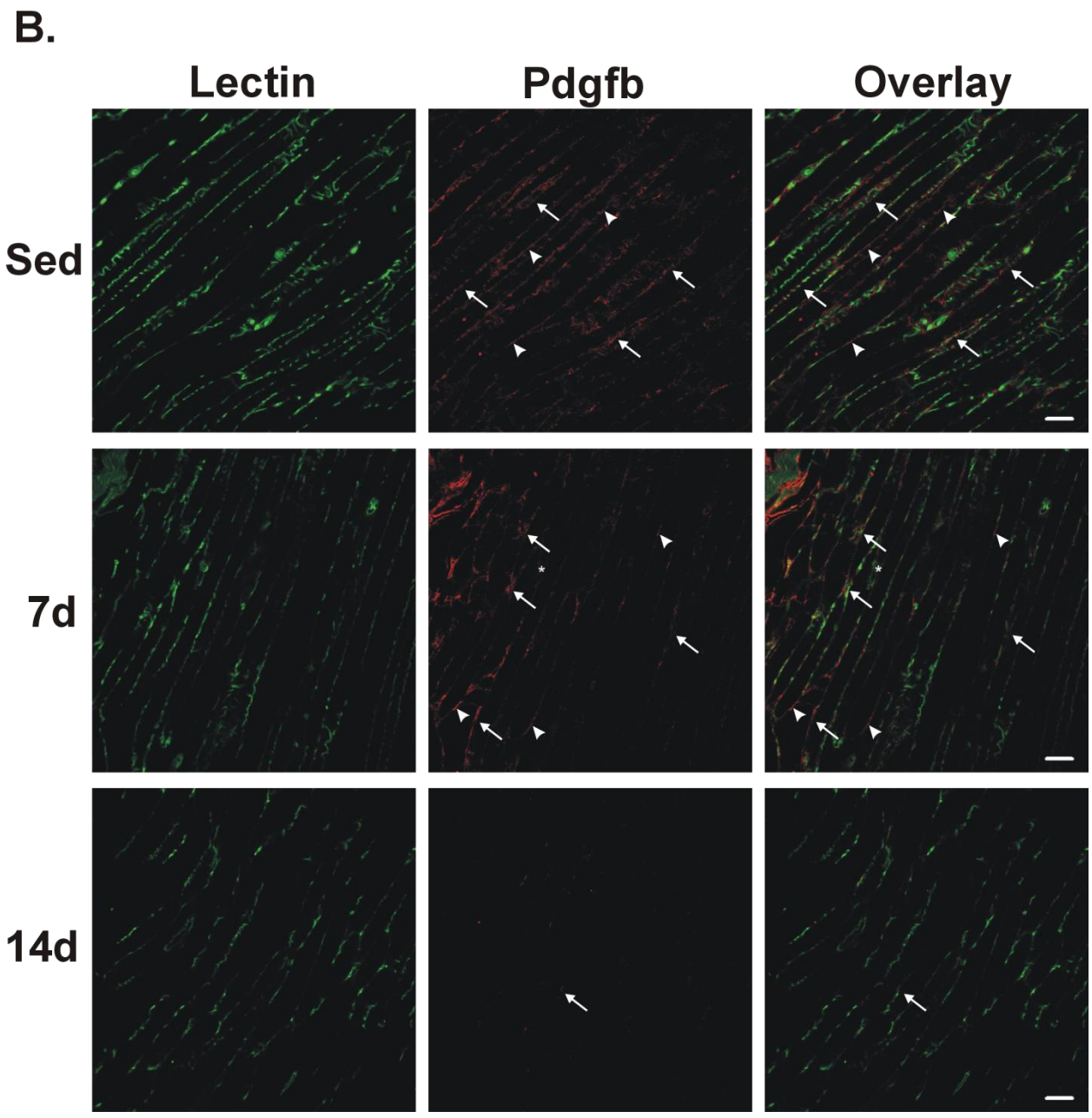


Figure 3-9. Pdgfb expression in cross-sections is stronger in trained compared to sedentary muscles.
Muscle cross-sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Pdgfb (red), and isolectin for vascular EC (green). Images were viewed with 63x objective. Pdgfb positive staining was observed to overlap with isolectin (arrow). Pdgfb staining that does not overlap isolectin (arrowhead). (Bar = 50μm).


A.
Lectin
Pdgfb
Overlay
Sed
7d
14d


B.
Lectin
Pdgfb
Overlay
Sed
7d
14d

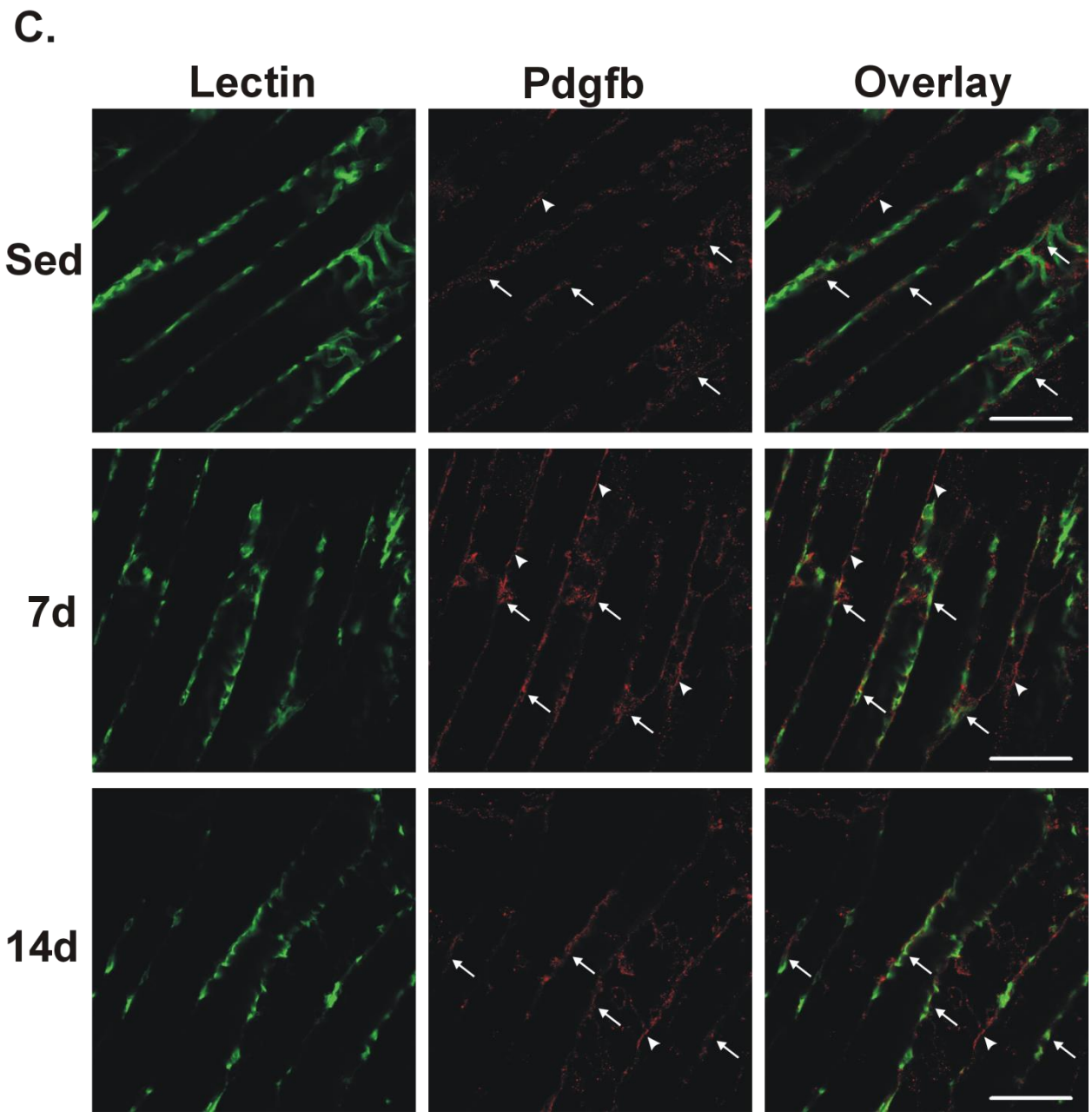

C.
Lectin
Pdgfb
Overlay
Sed
7d
14d

Figure 3-10. Pdgfb is not a definitive marker of tip cells in skeletal muscles.
Longitudinal sections from sedentary, 7d, and 14d trained mouse plantaris muscle were immunostained for Pdgfb (red), and isolectin for vascular EC (green). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. Pdgfb positive staining was observed to overlap with isolectin (arrow) and could also be observed in large vessels (asterisk). Pdgfb staining that does not overlap isolectin (arrowhead). (Bar = 50μm).

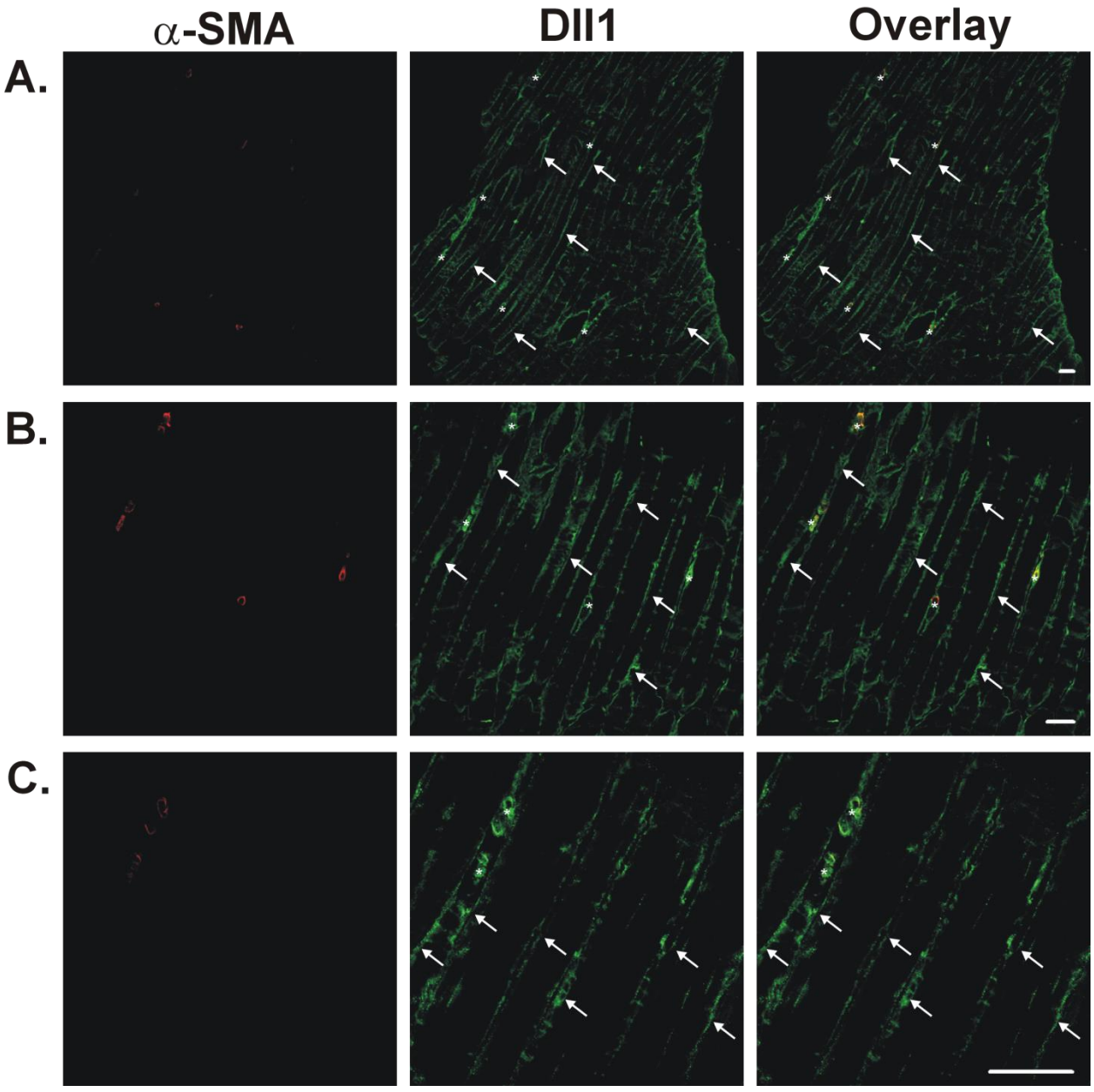


Figure 3-11. Dll1 is not expressed in mural cells in skeletal muscle with exercise training. Longitudinal sections from 7d trained mouse plantaris muscle were immunostained for Dll1 (green), and alpha smooth muscle actin (red). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. Dll1 positive staining (arrow). Dll1 positive staining was not observed in mural cells (asterisk). (Bar = 50μm).

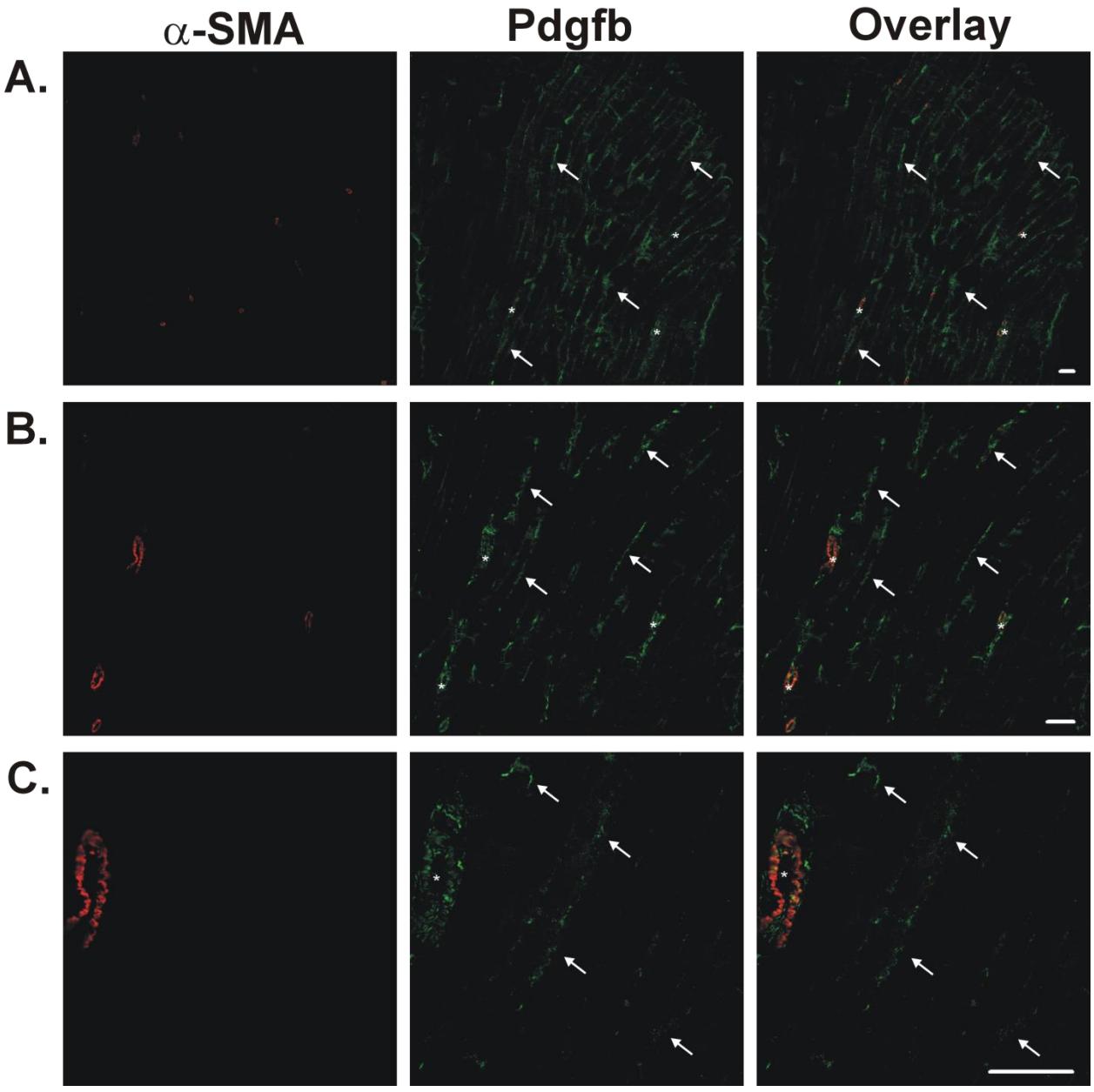
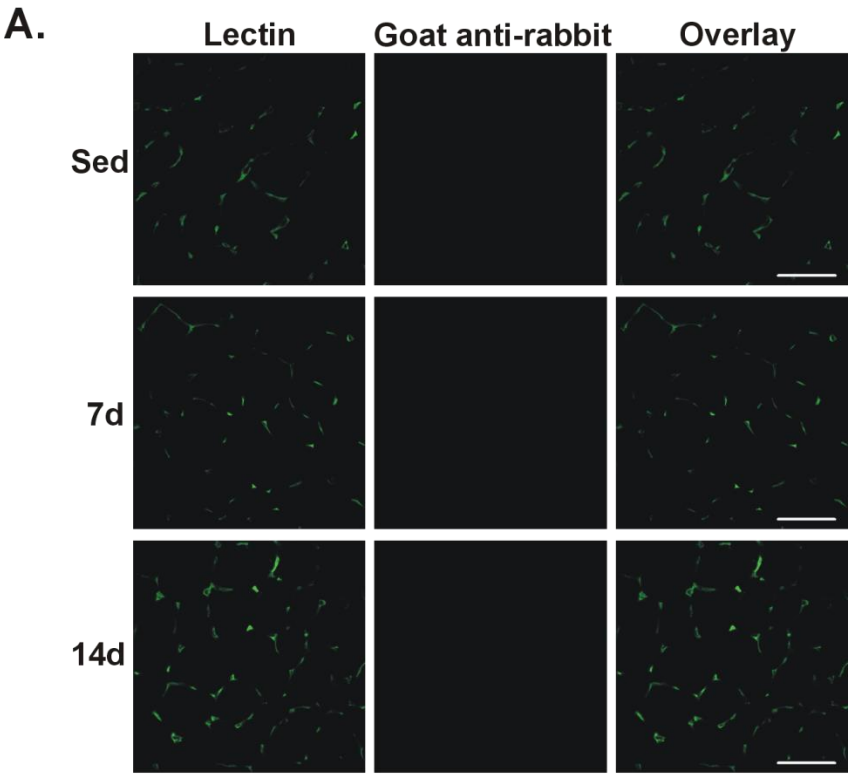


Figure 3-12. Pdgfb is not expressed in mural cells of skeletal muscle with exercise training. Longitudinal sections from 7d trained mouse plantaris muscle were immunostained for Pdgfb (green), and α smooth muscle actin (red). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. Pdgfb positive staining (arrow). Pdgfb positive staining was not observed in mural cells (asterisk). (Bar = 50μm).


A.
Lectin
Goat anti-rabbit
Overlay
Sed
7d
14d

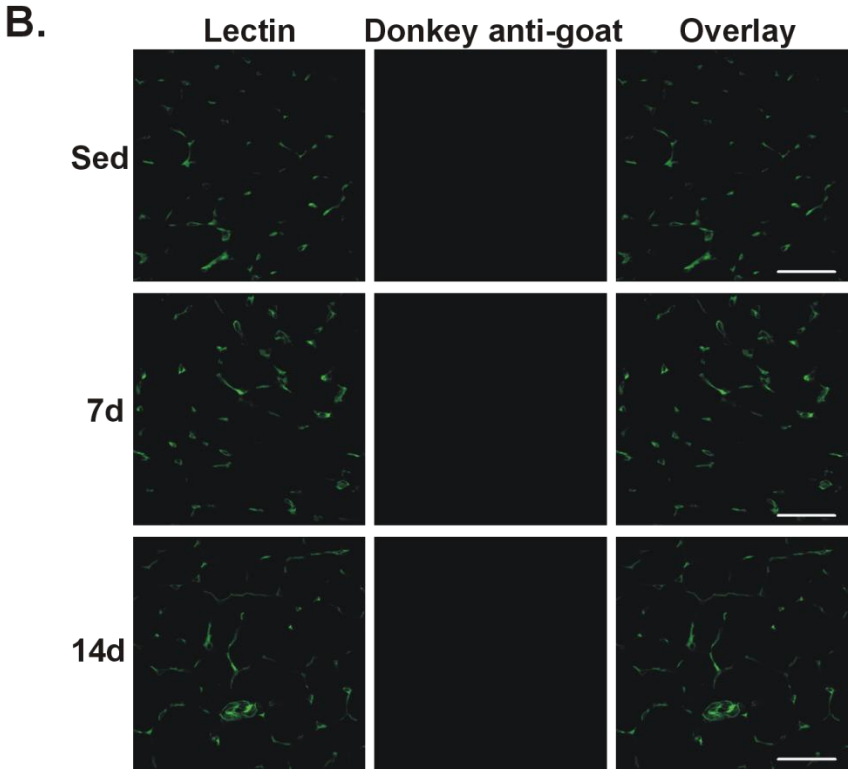

B.
Lectin
Donkey anti-goat
Overlay
Sed
7d
14d

Figure 3-13. Negative control showed no positive staining of species specific serum within muscle cross-section or the vasculature.
Cross-sections from Sed, 7d, and 14d trained mouse plantaris muscle were immunostained for goat serum (red) (A), or donkey serum (red) (B), and counterstained with isolection for vascular EC (green). Images were viewed with 63x objective. No positive signal was observed when omitting the species specific serum respected to the host of the secondary antibody. (Bar = 50μm).

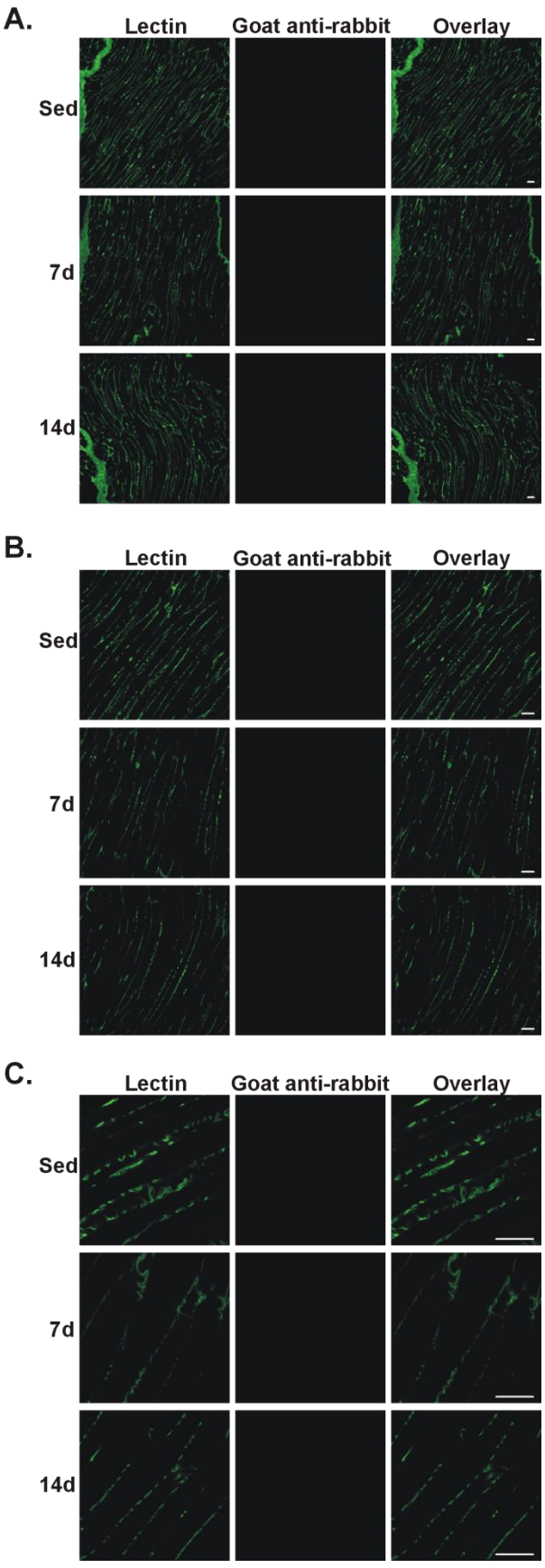

A.
Lectin
Goat anti-rabbit
Overlay
Sed
7d
14d
B.
Lectin
Goat anti-rabbit
Overlay
Sed
7d
14d
C.
Lectin
Goat anti-rabbit
Overlay
Sed
7d
14d

Figure 3-14. Negative control showed no positive staining of goat serum within longitudinal muscle section or the vasculature.
Longitudinal sections from Sed, 7d, and 14d trained mouse plantaris muscle were immunostained for goat serum, and isolection for vascular EC (green). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. No positive signal was observed when omitting the species specific serum respected to the host of the secondary antibody. (Bar = 50μm).

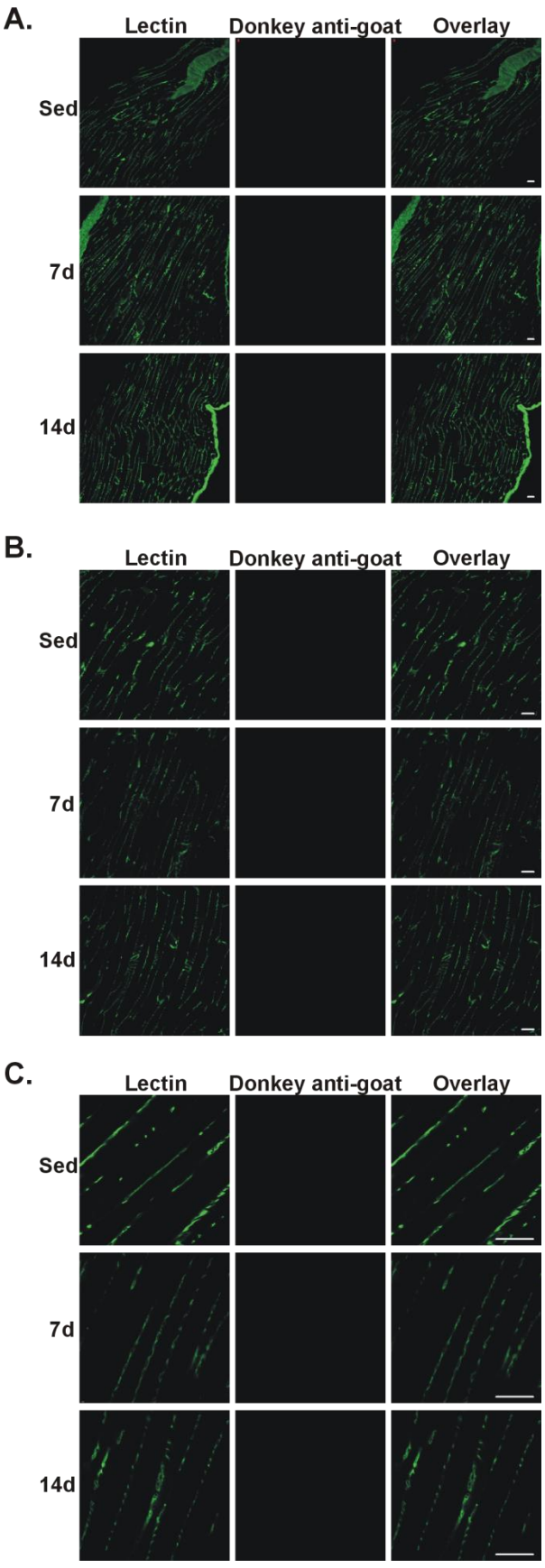

A.
Lectin
Donkey anti-goat
Overlay
Sed
7d
14d
B.
Lectin
Donkey anti-goat
Overlay
Sed
7d
14d
C.
Lectin
Donkey anti-goat
Overlay
Sed
7d
14d

Figure 3-15. Negative control showed no positive staining of donkey serum within longitudinal muscle section or the vasculature.
Longitudinal sections from Sed, 7d, and 14d trained mouse plantaris muscle were immunostained for donkey serum (red), and isolection for vascular EC (green). Images were viewed with 10x (A), 20x (B), and 63x (C) objectives. No positive signal was observed when omitting the species specific serum respected to the host of the secondary antibody. (Bar = 50μm).

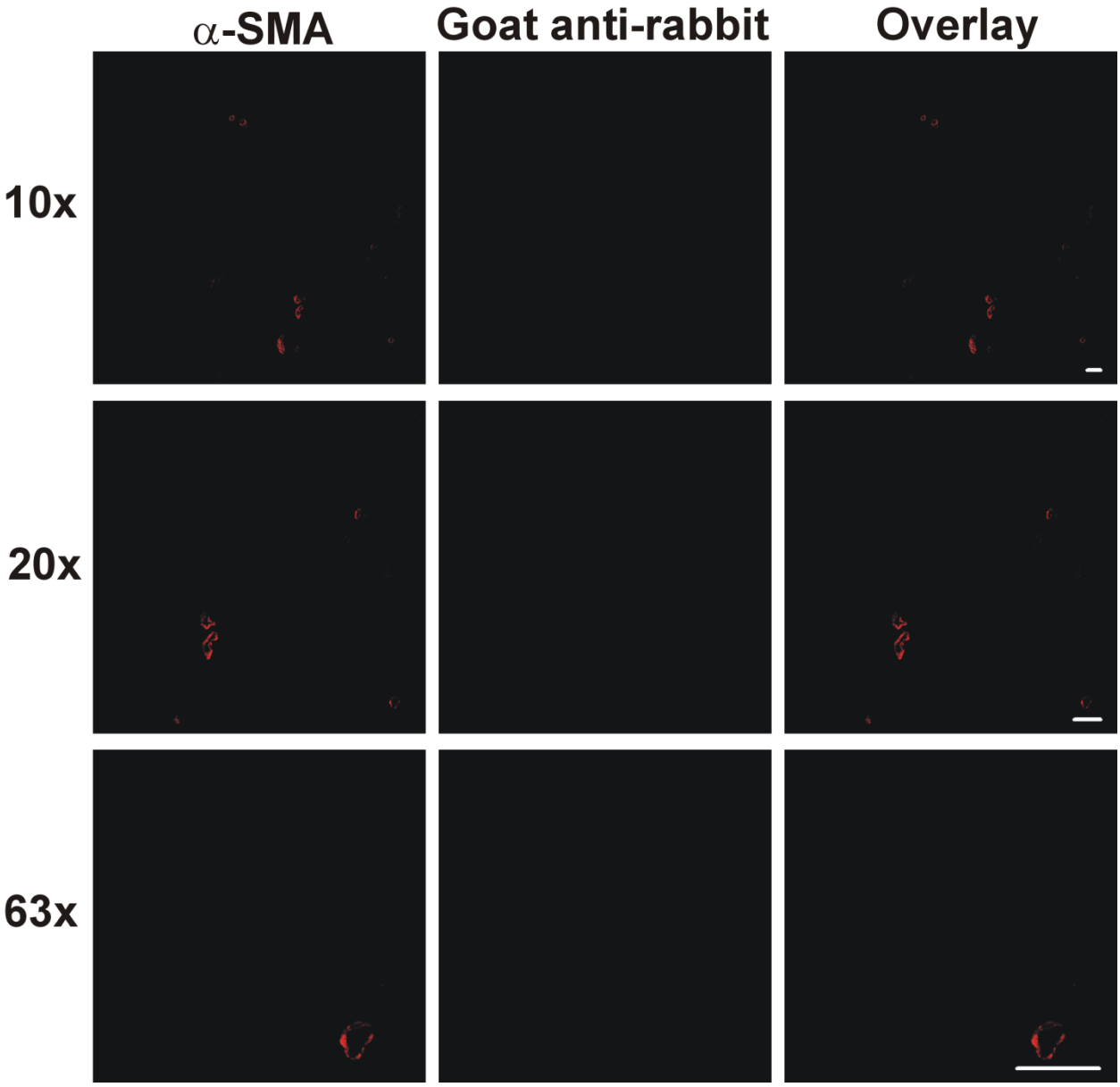


Figure 3-16. Negative control showed no positive staining of goat serum within longitudinal muscle section or mural cells.
Longitudinal sections from Sed, 7d, and 14d trained mouse plantaris muscle were immunostained for goat serum (green), and α smooth muscle actin (red). Images were viewed with 10x, 20x, and 63x objectives. No positive signal was observed when omitting the species specific serum respected to the host of the secondary antibody. (Bar = 50μm).

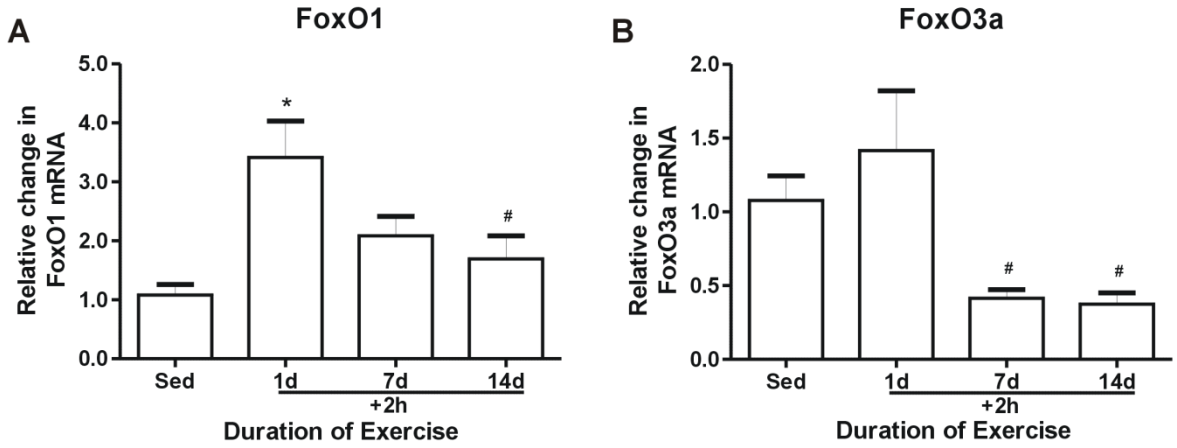


Figure 3-17. Exercise training elicited temporal changes in FoxO1 and FoxO3a expression.
Mice trained for 1, 7, 14 day (1d, 7d, 14d) or remained sedentary (Sed). Muscles were extracted 2 hours after training. (A) FoxO1 (n=6 per group) and (B) FoxO3a (n=6 per group) transcript levels were analyzed by qPCR. Values are presented as mean ± SEM. Significant training effect (p<0.05) (one way ANOVA) *=p<0.05 vs. Sedentary, #=p<0.05 vs. 1d day.

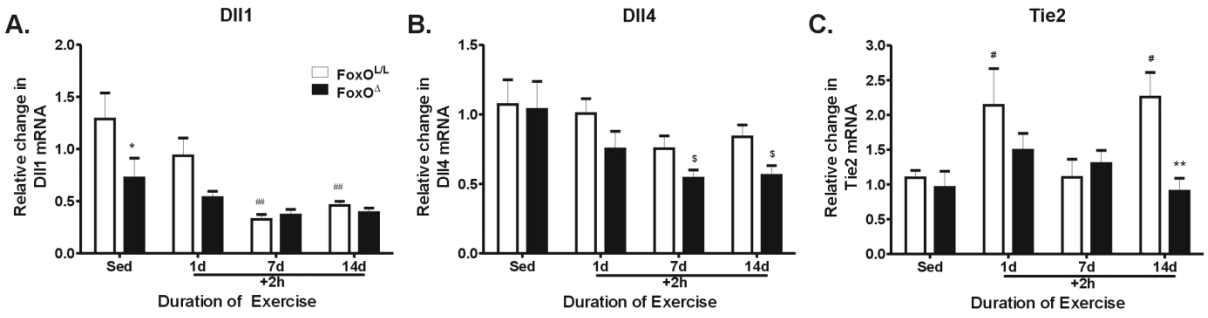


Figure 3-18. Long-term training mRNA analysis of Dll1, Dll4, and Tie2 expression in the FoxO$^{\Delta}$ model.

FoxO$^{L/L}$ and FoxO$^{\Delta}$ trained for 1, 7, 14 days (1d, 7d, 14d), or remained sedentary (Sed). Muscles were collected 2 hours after exercise. (A) Dll1 gene expression (B) Dll4 gene expression (C) Tie2 gene expression. Changes in Dll1, Dll4, and Tie2 mRNA expression were assessed by real time PCR. #=p<0.05 vs. FoxO$^{L/L}$ Sedentary, ##=p<0.001 vs. FoxO$^{L/L}$ Sedentary, $=$p$<0.05 vs. FoxO$^{\Delta}$ Sedentary, *=p<0.05 vs. Time-matched FoxO$^{L/L}$ mice, **=p<0.001 vs. time-matched FoxO$^{L/L}$ mice, 2-way ANOVA. (n=6/FoxO$^{L/L}$ group, n=5 for FoxO$^{\Delta}$ 1day training, n=6 for FoxO$^{\Delta}$ Sedentary, 7day, and 14day training).

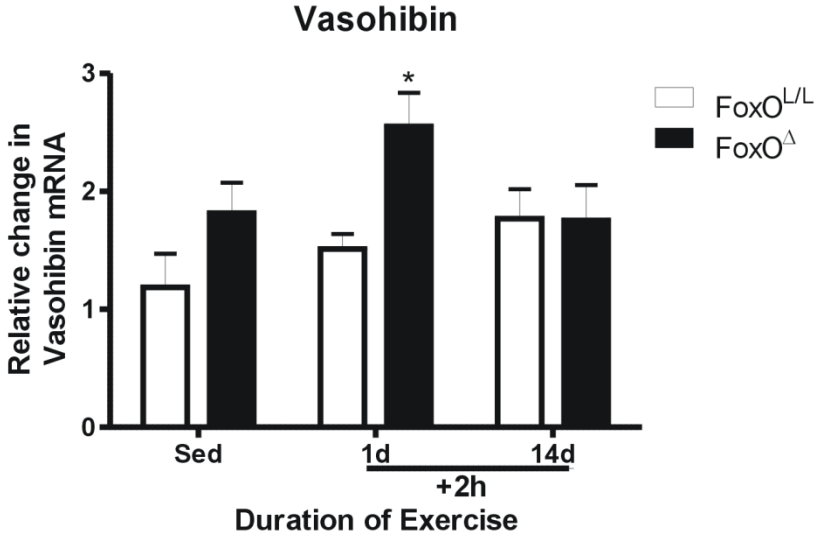


Figure 3-19. Long-term training mRNA analysis of Vasohibin expression in the FoxO$^{\Delta}$ model.

FoxO$^{L/L}$ and FoxO$^{\Delta}$ trained for 1, 14 days (1d, 14d), or remained sedentary (Sed). Muscles were collected 2 hours after exercise. Changes in vasohibin mRNA expression were assessed by real time PCR. *=p<0.05 vs. Time-matched FoxO$^{L/L}$ mice, 2-way ANOVA. (n=6/FoxO$^{L/L}$ group, n=5 for FoxO$^{\Delta}$ 1day training, n=6 for FoxO$^{\Delta}$ Sedentary, and 14day training).

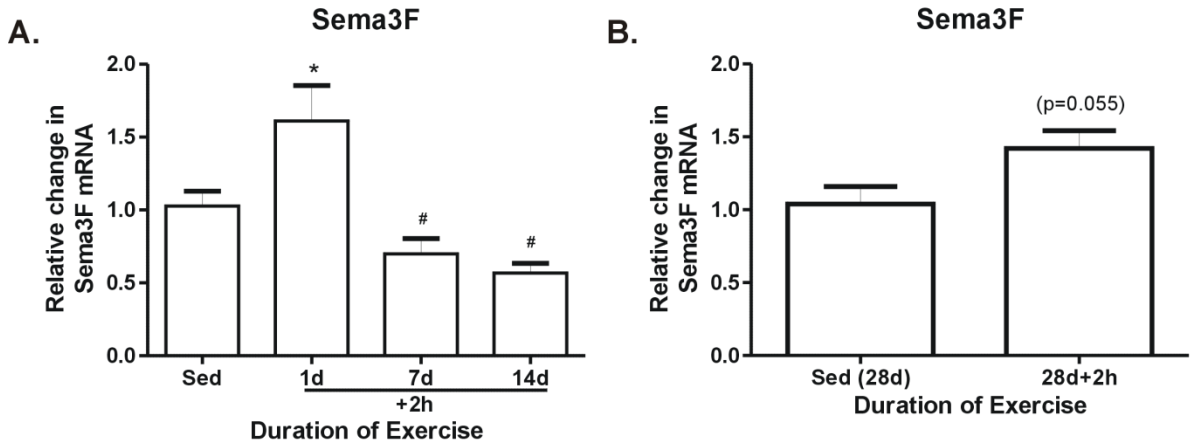


Figure 3-20. Long-term and extended training mRNA analysis of Sema3F expression.
FoxO$^{L/L}$ mice were trained for 1, 7, 14, 28 days (1d, 7d, 14d, 28d), or remained sedentary (Sed). Muscles were collected 2 hours after exercise. Changes in Sema3F mRNA expression were assessed by real time PCR.*=p<0.05 vs. Sedentary, #=p<0.05 vs. 1day training, 1-way ANOVA. (n=6/ group) (B) Sema3F gene expression. 1-way ANOVA. (n=6 for sedentary group, n=5 for 28day training).

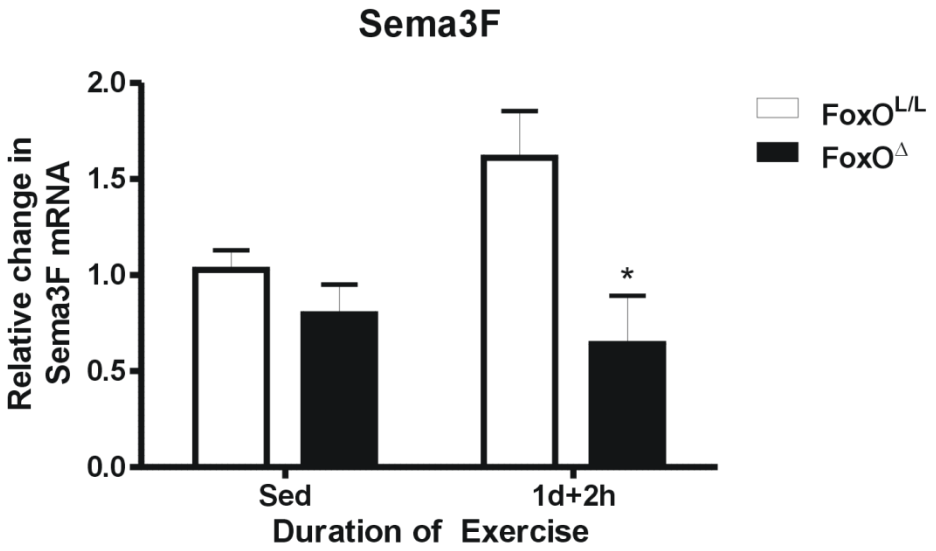


Figure 3-21. Acute training mRNA analysis of Sema3F in the FoxO$^{\Delta}$ model.

FoxO$^{L/L}$ and FoxO$^{\Delta}$ performed an acute bout of exercise (1d), or remained sedentary (Sed). Muscles were collected 2 hours after exercise. Changes in Sema3F mRNA expression were assessed by real time PCR. *=p<0.05 vs. Time-matched FoxO$^{L/L}$ mice, 2-way ANOVA. (n=6/FoxO$^{L/L}$ group, n=5 for FoxO$^{\Delta}$ 1day training).

Chapter 4: Discussion

The principle contribution of this study is to demonstrate the expression patterns of key molecules that are involved in endothelial sprout formation and maturation in mouse skeletal muscle during exercise. As shown with immunohistochemistry, both Dll1 and Dll4 proteins were down-regulated with repeated bouts of aerobic exercise. Tie2 protein was expressed in skeletal muscle, but its expression was not changed with exercise. Pdgfb was present in the skeletal muscle endothelium, but its expression was not limited to tip cells as previous studies have demonstrated in zebrafish embryo and mouse retina. Changes in tip cell gene expression in response to exercise were also assessed, but discrepancies were found between mRNA and protein levels. Furthermore, we have shown that FoxO transcription factors play a role in regulating the transcript levels of Dll1, Dll4, Tie2, and anti-angiogenic factors such as vasohibin and Sema3F in skeletal muscle during aerobic exercise.

Acute and short term exercise can elicit temporal expression of several angiogenic factors (Gavin & Wagner, 2001; Olenich et al., 2013). VEGF is known to be highly expressed during the early phases of an exercise regimen and diminishes as training continues (Breen et al., 1996; Lloyd et al., 2003). Elevation in Tie2 mRNA and Ang2-to-Ang1 ratio were also observed at the early time points and attenuated later in training (Lloyd et al., 2003). Repeated exercise training elicits effects such as inducing the expansion and remodeling of the vasculature, and promoting stabilization of the newly expanded capillary network, commonly seen at 7 and 14 days of exercise, at which point capillary density and capillary-to-fibre ratio have been reported to increase

(Olenich et al., 2013, Slopack et al., manuscript in progress). Beyond this, very little is known about the process of endothelial cell sprouting in skeletal muscle. In order to identify tip cells and mature capillaries, I first examined the mRNA levels of several targets that have been previously established to be associated with the formation of capillary sprouts and vascular maturation. This was then followed by *in vivo* assessment using immunohistochemistry in order to observe changes in endothelial cell morphology and the localization of tip cell targets in skeletal muscle.

Dll4/Notch signaling regulates the formation of tip cell during angiogenesis. Dll4 mRNA was decreased (p=0.06) at day 5 of exercise compared to sedentary (Fig. 3-1B). The tendency of transcript reduction seen with Dll4 at this time point may allow for augmented tip cell phenotype selection without the interference of lateral inhibition. Evidence has shown that the expression of Dll4 is upregulated by VEGF (Blanco & Gerhardt, 2012); hence, the decline in VEGF expression with repeated exercise as previously reported by other researchers (Gavin & Wagner, 2001; Lloyd et al., 2003) may contribute to the downregulation of Dll4. Both muscle cross-sections and longitudinal sections displayed a broad expression of Dll4 in the skeletal muscle endothelium, based on the overlap with isolectin staining. Interestingly, Dll4 signals were also seen in some areas that were not isolectin positive, particularly in areas between two parallel capillaries (Fig. 3-4C). This area of enriched Dll4 protein may indicate an early sign of the formation of lateral branches between adjacent vessels. Other targets such as VEGFR3 that have previously been identified in tip cells (Tammela et al., 2011) should be investigated, as the isolectin staining did not appear to identify the extension of filopodia in skeletal muscle capillaries. The expression of Dll4

can also be seen in large vessels (Fig 3-3, 3-4C). Perhaps Dll4 serves similar functions as Dll1 in maintaining arterial phenotype in skeletal muscles. The decrease in Dll4 protein expression seen at 7 days and 14 days of exercise compared to sedentary (Fig. 3-3, 3-4) may suggest a state of enhanced endothelial sprouting, as lateral inhibition is subdued.

Dll1 was previously reported to be involved with tip cell selection and a regulator of arterial maintenance (Limbourg et al., 2007; Napp et al., 2012). Dll1 expression during treadmill running was transient, increasing at day 1 and day 3 and returned to basal levels on the fifth day (Fig. 3-1A). This may suggest that the commencement of tip cell selection occurs at the early stages of the exercise regimen. This increase in Dll1 expression is also immediate, as 2 hours of recovery following an acute bout of exercise is sufficient to return Dll1 mRNA back to basal levels. With repeated exercise, Dll1 is down-regulated in skeletal muscles at time points when initiation of capillary growth would be expected to occur with training (Fig. 3-18A). Dll1 had previously been identified in arterial endothelial cells, where its expression is crucial for vascular maintenance during fetal development (Sörensen et al., 2009). Our mRNA analyses suggest that the role of Dll1 may differ in adults, where the suppression of Dll1 is required for angiogenesis to occur during repeated exercise. Recent findings from Adam and colleagues have found that Dll1 signaling is promoted in human umbilical vein endothelial cells when a quiescent endothelial phenotype is required (Adam et al., 2013). With this concept in mind, the decrease in Dll1 at day 7 and 14 of training would correspond to the downregulation of Delta-Notch signaling in order to promote capillary growth. Similar to Dll4, Dll1 expression overlaps isolectin staining (Fig. 3-5, 3-6), and

also is localized to areas between adjacent vessels that were not isolectin positive (Fig. 3-6). This further validates the concept of early development of lateral branches as this area of the endothelium is undergoing a pre-sprouting state of tip cell selection. This selection process may slow down in skeletal muscle at 14 days of exercise, as Dll1 protein expression is decreased compared to sedentary and 7 days of training. Dll1 immunostaining did not overlap with α smooth muscle actin (Fig. 3-11), indicating that it is not expressed within peri-endothelial cells of skeletal muscle.

Tie2 expression in the endothelium has been shown to be associated with the recruitment of pericytes and smooth muscle cells which influence vessel architecture (Patan, 1998; Peters et al., 2004). The decrease in Tie2 mRNA at day 5 of exercise (Fig. 3-1C) may indicate the starting point at which capillary destabilization occurs in order to initiate morphological changes in the endothelium in response to repeated exercise. Increases in Tie2 mRNA with treadmill running at 1 and 14 days are consistent with its role in capillary stabilization (Fig. 3-18B), both before and following the formation of endothelial sprouts, respectively. Interestingly, our findings showed Tie2 protein expression is present in the capillaries, but the intensity of the signal remained unchanged with repeated exercise bouts compared to sedentary (Fig. 3-7, 3-8). I also stained cultured mouse skeletal muscle endothelial cells (mSMECs) with isolection and the Tie2 antibody to validate the presence of Tie2. The Tie2 signal overlapped isolectin, thus we can conclude that the antibody is suitable for identifying Tie2 expression in murine endothelial cells. Technical issues such as antibody concentration and incubation time may need to be adjusted in order to properly assess Tie2 expression in muscle sections.

In other models, Pdgfb was shown to be a marker of tip cells (Hellström et al., 2007). We have examined Pdgfb in mouse skeletal muscles and found that its expression extensive throughout the microvascular endothelium and is not limited to tip cells. Pdgfb signal from immunostaining of muscle cross- (Fig. 3-9) and longitudinal (Fig. 3-10) sections overlapped isolectin, suggesting that it is expressed on the surface of endothelial cells. Pdgfb protein was also identified in areas without positive isolectin staining (Fig. 3-10). These signals that were not overlapped with isolectin were seen in-line and positioned superior and inferior of isolectin positive capillaries (Fig. 3-10C). Similar to Dll1, the Pdgfb signal was not found in mural cells (Fig. 3-12), suggesting its expression is specific to the surface of small vessels in the skeletal muscle endothelium. The retention motif of the Pdgfb gene is structurally similar to the VEGF-A heparin sulfate interacting domain (Ostman et al., 1991); hence, Pdgfb signals that were in-line, but did not overlap with isolectin stain may represent secreted Pdgfb proteins that were bound to heparin sulfate rich proteins, such as the extracellular matrix. More work will be required to decipher this phenomenon.

We recently demonstrated the effects of FoxO transcription factor by use of the MxCre:FoxO1, 3, 4 transgenic mouse model. Conditional deletion of FoxO 1, 3a and 4 in skeletal muscle endothelial cells attenuated the angiostatic properties of FoxO, resulting in accelerated capillary growth during exercise (Slopack et al., manuscript in progress). We hypothesized that gene targets involved in vessel sprouting contributed to this occurrence. Basal Dll1 transcript levels were downregulated in the FoxO transgenic animals, but neither Dll1 or Dll4 mRNA was altered with 7-day and 14-day of training in $FoxO^{\Delta}$ mice (Fig. 3-18A, 3-18B), suggesting that the Delta/Notch signaling

pathway is not regulated by FoxO during repeated exercise. Furthermore, we observed the loss of the exercise response in Tie2 expression in the FoxO$^{\Delta}$ model. However, our data reveal that Tie2 mRNA level is not regulated by FoxO at rest. While most of the changes fit in accordance with the alteration in FoxO protein, this is not apparent for Tie2 mRNA level at 14-day as FoxO protein is greatly reduced at that time point (Slopack et al., manuscript in progress). Tie2 expression at 14-day appears to be strongly dependent on FoxO. Although it is not a direct connection, the expression of Tie2 at this time point may occur indirectly through other genes that are influenced by FoxO. More work will be required to decipher this event during repeated aerobic exercise.

Our lab has recently shown that 2 hours following a single bout of exercise can elicit an increase in FoxO1 and FoxO3a transcript (Fig. 3-17A) and protein levels (Slopack et al., manuscript in progress), and that FoxO proteins regulated the expression of anti-angiogenic factor TSP-1 at that time point (Slopack et al., manuscript in progress). This led us to believe that temporal changes in FoxO1 and FoxO3a in response to an acute exercise bout may influence the expression of additional downstream targets that act to repress an angiogenic response. Here, we look at the transcription of VASH-1 as it has previously been shown to share similar expression patterns as TSP-1 during short term exercise (Kishlyansky et al., 2010). Kishlyansky et al. observed an immediate increase in VASH-1 protein in skeletal muscle following acute exercise, while VASH-1 protein returned to basal level by 4 hours of recovery. We observed no change in VASH-1 mRNA levels following exercise with 2 hours recovery compared to sedentary animals (Fig. 3-19). It is possible that we would see increases in

VASH-1 mRNA if we assessed muscle immediately after the completion of exercise. The inconsistency seen between VASH-1 mRNA and protein levels may be due to an increased rate of protein translation, or that VASH-1 protein is required for structural maintenance in the skeletal muscle endothelium, thus it is less likely to be targeted for degradation. Alternatively, VASH-1 expression was elevated in $FoxO^{\Delta}$ animals trained for 1 day plus 2 hours of recovery (Fig. 3-19). This upregulation may assist in compensating for the loss of FoxO, in continuing to provoke angiostatic signals to the endothelium. The negative regulation of VASH-1 by FoxO suggests that FoxO is able to suppress its downstream targets in coordinating the rate of which angiogenesis occurs.

Sema3F is expressed in endothelial cells and elicits anti-angiogenic properties, but the factors that regulate its expression are not known. I found that the expression patterns of Sema3F in skeletal muscle were very similar to that of FoxO1, FoxO3a, and TSP-1 in response to aerobic exercise (Fig. 3-20), leading to the hypothesis that FoxO transcription factors govern the expression of Sema3F during exercise. We examined Sema3F levels in $FoxO^{\Delta}$ animals at the 1 day of exercise plus 2 hours of recovery, as FoxO1 and FoxO3a proteins were shown to be significantly elevated at the time point. The increase in Sema3F seen at 1d+2h of exercise was abolished in $FoxO^{\Delta}$ animals (Fig. 3-21), demonstrating that its expression is regulated by FoxO transcription factors. Basal levels of Sema3F were unchanged in $FoxO^{\Delta}$ mice compared to $FoxO^{L/L}$. This suggests that FoxO is a key player in coordinating cellular events, including the regulation of multiple anti-angiogenic targets during exercise.

Chapter 5: Conclusions and Future Perspectives

This study provides novel insight into the mechanisms behind sprouting angiogenesis by examining genes related to tip cell formation in a model that has never been studied before for this purpose. We have contributed to the understanding of the physiology behind the initiation of capillary growth in skeletal muscle induced by exercise. This is of particular interest for exploring targets stimulated by exercise therapy to promote the enhancement of blood flow in ischemic tissues. Our findings from the mRNA analysis and immunohistochemistry suggest that Dll1 and Dll4 are regulated in skeletal muscle during aerobic exercise. Tie2 is also shown to be regulated at the transcription level with exercise, and potentially may play a role in the maturation of the vascular network. The expression of these genes is altered at different exercise time-points which suggest their role in optimizing the initial periods of capillary sprouts, and shaping the foundation of the upcoming vascular network. The analysis of an exercise time-course, in conjunction with the use of the $FoxO^{\Delta}$ mice provided understanding of the regulatory role that FoxO plays in coordinating the expression of pro- and anti-angiogenic factors in skeletal muscle during exercise.

Immunostaining of longitudinal muscle sections allowed us to visualize the length of the capillary network and the formation of lateral branches, which is believed to be an ideal approach to study endothelial sprouts induced by exercise. I expected to be able to identify tip cells in these muscle sections by using known markers, but the targets that I chose were not localized specifically to the filopodia of the capillary in skeletal muscle. Identifying the appropriate protein to be used as a tip cell marker is essential in

the continuation of studying vessel sprouts in this model. An objective approach in quantifying the strength of the immunostaining signal in muscle sections by using quantitative software is one method to provide an accurate representation of the protein expression. Quantitative protein analysis could also be used to provide a more accurate assessment of gene expression in skeletal muscle to reduce the discrepancies seen between mRNA assessments and immunostaining. *In vitro* analsysis of cell migration may also be suitable in determining the key players of sprout formation, and provide further understanding of these known targets in skeletal muscle endothelial cells. Other molecules such as VEGFR3 and Sema3A have previously been implicated in angiogenic sprouting in mouse retina (Kim et al., 2011; Tammela et al., 2008). They may potentially be candidates for the identification and regulation of tip cells in skeletal muscle. Although the exercise time-course of 1 to 14 days could be used to map out the occurrence of molecular and morphological responses in vascular remodeling during different phases of training, the current and previous studies have shown that the period of recovery following exercise can induce a dramatic change in gene expression. Broadening the time-course to include pre-exercise time periods would allow us to further pinpoint the expression of the target of interest. Utilizing siRNA or a Notch inhibitor in *in vitro* experimentation or a transgenic animal model of conditional deletion to suppress the gene expression of Dll1 and/or Dll4 may provide mechanistic approaches in exploring the role of Delta/Notch signaling in skeletal muscle endothelium.

Future studies should continue to explore downstream targets of FoxO in identifying its role in coordinating the balance between angiogenic and angiostatic

factors in response to different stimuli. The expression pattern and localization of tip cell targets could also be examined in human subjects undergoing exercise. These observations could motivate future analysis in optimizing exercise training protocols that would improve the skeletal muscle vascularisation of peripheral artery disease patients.

Appendix

In my first year of the masters program, I was involved with three collaborative projects which focused on 1) the mechanisms of corticosterone in mediating capillary growth, 2) the role of Angiotensin II in inducing angiogenic signals within skeletal muscle, and 3) the involvement of FoxO1 and FoxO3a in regulating exercise induced angiogenesis. The following is a summary of my contribution to the three manuscripts.

Inhibition of proliferation, migration and proteolysis contribute to corticosterone-mediated inhibition of angiogenesis (Shikatani, et al., 2012)

Study objective

Corticosterone is an endogenous glucocorticoid in rodents. It is well established that pharmacological levels of glucocorticoid steroids promotes capillary regression, and prevents vascular development (Folkman et al., 1983; Small et al., 2005). However, the angiostatic effects of pathophysiological elevation of glucocorticoid are not known. The objective of this study is to assess the cellular mechanisms of pathophysiological concentration of corticosterone in the inhibition of angiogenesis.

Results

Pure corticosterone pellets implanted subcutaneously in rats resulted in elevated basal plasma cortiosterone levels, which reduced capillary-to-fibre ratio in the tibialis anterior muscle compared to control rats. Capillary segments in 3D culture treated with 600nM of corticosterone for 48h resulted in decrease in capillary sprouting compared to

control. Rat skeletal muscle endothelial cells treated with corticosterone had reduced VEGF mRNA levels and decreased proliferation rate compared to control. Scrape migration assay reveled that corticosterone reduced endothelial migration distance. This is likely contributed by the reduction in MMP-2 levels with corticosterone, as both total MMP-2 and activated MMP-2 levels were reduced in endothelial cells treated with corticosterone for 48h compared to control. Corticosterone mediates the inhibition of angiogenesis by interfering with endothelial cell proliferation, migration, and proteolysis.

Project contribution

I was involved in the skeletal muscle endothelial cell culture experiments that displayed cell rounding with corticosterone treatment (Figure A-1 D/E), as well as, performed and captured the images of the endothelial cell migration assay (Figure A-2 A). I have also contributed to the assessment of endothelial cell apoptosis by immunostaining for cleaved caspase-3 (Figure A-3 A/B).

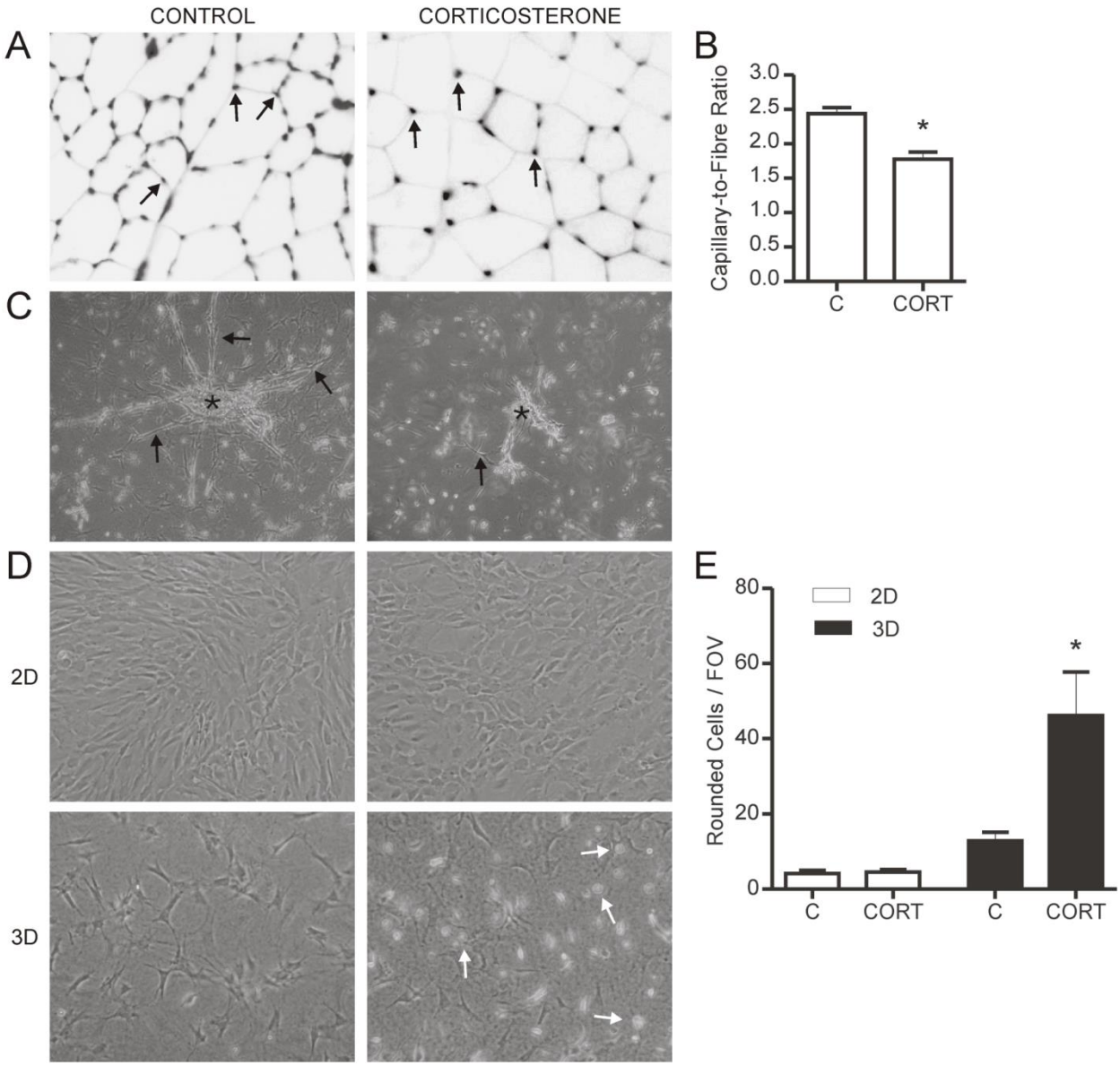


Figure A-1: Corticosterone inhibits angiogenesis *in vivo* and *ex vivo*.
Tibialis anterior muscles from rats implanted with either wax (Control) or corticosterone pellets (n = 5 per group) were sectioned and stained for capillaries using Griffonia Simplicifolia isolectin-fluorescein. Images have been inverted to enhance visualization of the muscle fibers. Arrows indicate capillaries (A). Capillary to fiber counts were made on 5 representative fields of view per rat (*p = 0.002, vs. control) (B). Isolated capillary segments were embedded in type I collagen and treated with 600 nM corticosterone for 72 hours. Capillary segments are indicated by an asterisk (*), while arrows indicate sprouts arising from the original segment (516magnification) (C). Skeletal muscle endothelial cells suspended in a 3-dimensional type I collagen culture treated with corticosterone displayed increased rounding of cells (indicated with white arrows), which was not evident in monolayer (2D) cultures (D, E *p = 0.04 vs 3D Control). C Control, CORT Corticosterone.

Shikatani, E. A., Trifonova, A., Mandel, E. R., Liu, S. T. K., Roudier, E., Krylova, A., Szigiato, A., Beaudry, J., Riddell, M. C., Haas, T. L. (2012). Inhibition of Proliferation, Migration and Proteolysis Contribute to Corticosterone-Mediated Inhibition of Angiogenesis. *PLoS ONE* 7(10): e46625.

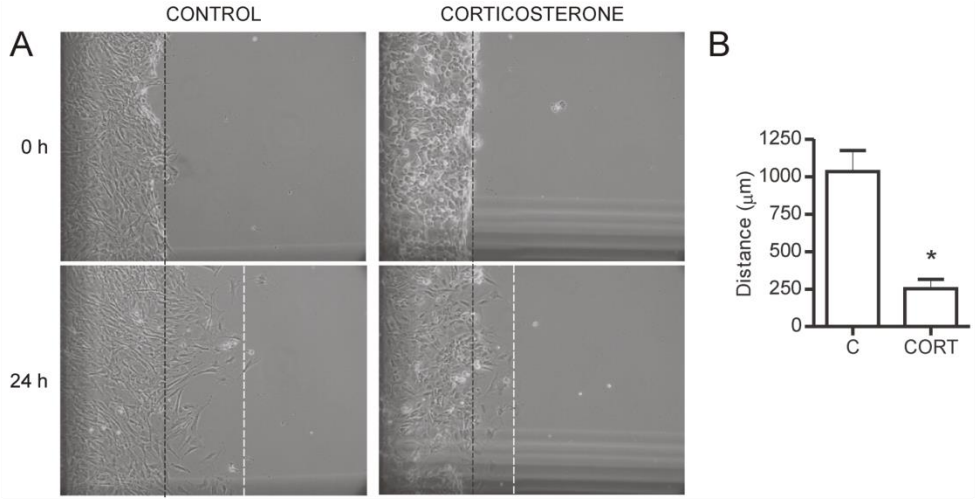


Figure A-2: Corticosterone inhibits endothelial cell migration.
Endothelial cells ($2.0x10^6$) were plated in 60 mm^2 plates coated with 1.5% gelatin and treated with 600nM corticosterone for 48 hours. Cells were pretreated with 5 μg/ml Mitomycin C for 2 hours prior to removing cells from half of the dish using a rubber cell scraper. Cells then were allowed to migrate into the scrape area for 24 hours (A). The dashed black line denotes the edge of the scrape at time 0, and the dashed white line denotes migration front after 24 hr. Migration distance was calculated as the difference between white and black lines in each field of view. Corticosterone inhibited migration of endothelial cells into the scrape area (**p*,0.0001 vs. control, n = 11 measurements from 34 fields of view) (B).

Shikatani, E. A., Trifonova, A., Mandel, E. R., Liu, S. T. K., Roudier, E., Krylova, A., Szigiato, A., Beaudry, J., Riddell, M. C., Haas, T. L. (2012). Inhibition of Proliferation, Migration and Proteolysis Contribute to Corticosterone-Mediated Inhibition of Angiogenesis. *PLoS ONE 7*(10): e46625.

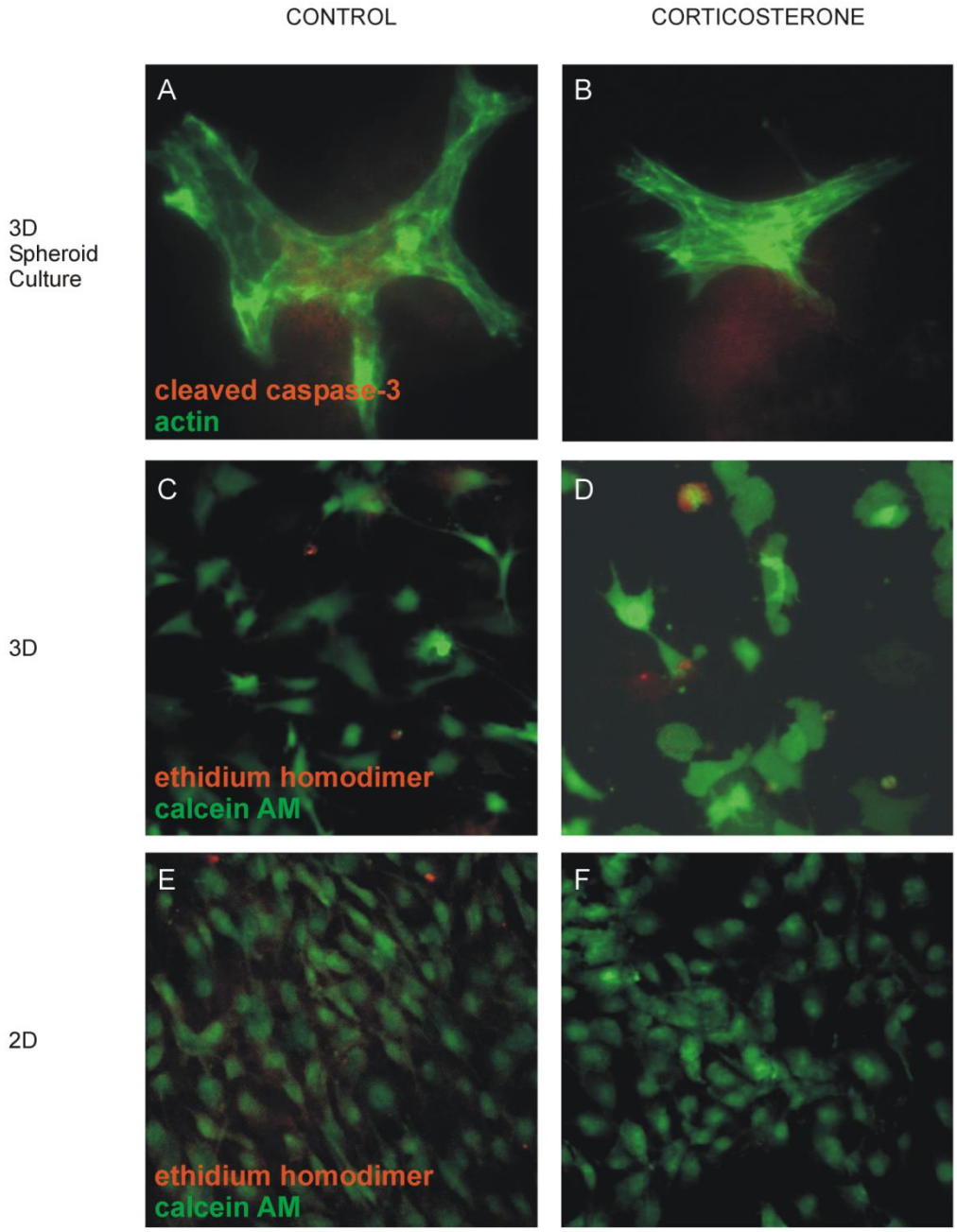


Figure A-3: Corticosterone does not cause apoptosis or necrosis of endothelial cells.
Skeletal muscle endothelial cell spheroids were suspended in a 3-dimensional type I collagen culture (A, B). Spheroids were treated for 48 hours with 600 nM corticosterone, prior to immunostaining for cleaved caspase-3 (red) and actin (green). Endothelial cells were either resuspended in 3D type 1 collagen ($5.0x10^5$ cells/ml) or in monolayer cultures ($1.0x10^6$ cells) plated in 35 mm^2 dishes coated in type-1 collagen and treated with 600 nM corticosterone for 48 hours (C-E). Cells were stained with a live/dead cell quantification kit (Molecular Probes). Live cells (green) were detectable in all conditions, while minimal dead cells (red) were visible in any condition.
C Control, CORT Corticosterone.

Shikatani, E. A., Trifonova, A., Mandel, E. R., Liu, S. T. K., Roudier, E., Krylova, A., Szigiato, A., Beaudry, J., Riddell, M. C., Haas, T. L. (2012). Inhibition of Proliferation, Migration and Proteolysis Contribute to Corticosterone-Mediated Inhibition of Angiogenesis. *PLoS ONE* 7(10): e46625.

Angiotensin II evokes angiogenic signals within skeletal muscle through coordinated effects on skeletal myocytes and endothelial cells (Gorman et al., 2013)

Study objective

Angiogenesis induced by skeletal muscle overload is contributed by the augmented expression of VEGF and MMP-2 (Rivilis et al., 2002; Williams et al., 2006). The upstream mediator of overload-induced expression of VEGF is not known. Angiotensin II (Ang II) is a regulator of the vasculature and is reported to modulate angiogenesis. This study demonstrates that Ang II promotes cross-talk between skeletal muscle fibres and endothelial cells, but that Ang II signaling is not required for overload-induced angiogenesis.

Results

The expression of angiotensinogen, a precursor of Ang II, is increased in overloaded extensor digitorum longus muscle. Ang II treatment stimulated the production of VEGF in myocytes and skeletal muscle endothelial cells. Ang II treatment also induced the phosphorylation of ERK1/2 in C2C12 myotubes and cultured endothelial cells. Blockade of AT1 receptor did not prevent capillary growth in response to muscle overload. Endothelial cells treated with both Ang II and AT1R blockade had increased VEGF mRNA levels compared to cells treated with Ang II alone. These results indicate that endogenous Ang II is involved with the production of VEGF by skeletal myocytes, but that Ang II is not required for overload-induced angiogenesis.

Project contribution

My contribution to this project includes performing the muscle overload and osmotic pump infusion surgeries on mice, the extraction of skeletal muscles and endothelial cell isolation from these animals, followed by the capillary-to-fibre assessment (Figure A-8), and mRNA (Figure A-4 A/B) and protein analysis (Figure A-4 C-E) of angiotensinogen, AT1R, and AT2R levels. I also measured Ang, AT1R, AT2R, p-ERK1/2 and p-Akt protein levels in both cultured endothelial cells (Figure A-5, Figure A-7) and C2C12 myotubes (Figure A-5, Figure A-6). Moreover, I performed various drug treatments to assess VEGF mRNA levels in cultured endothelial cells and C2C12 myotubes (Figure A-9).

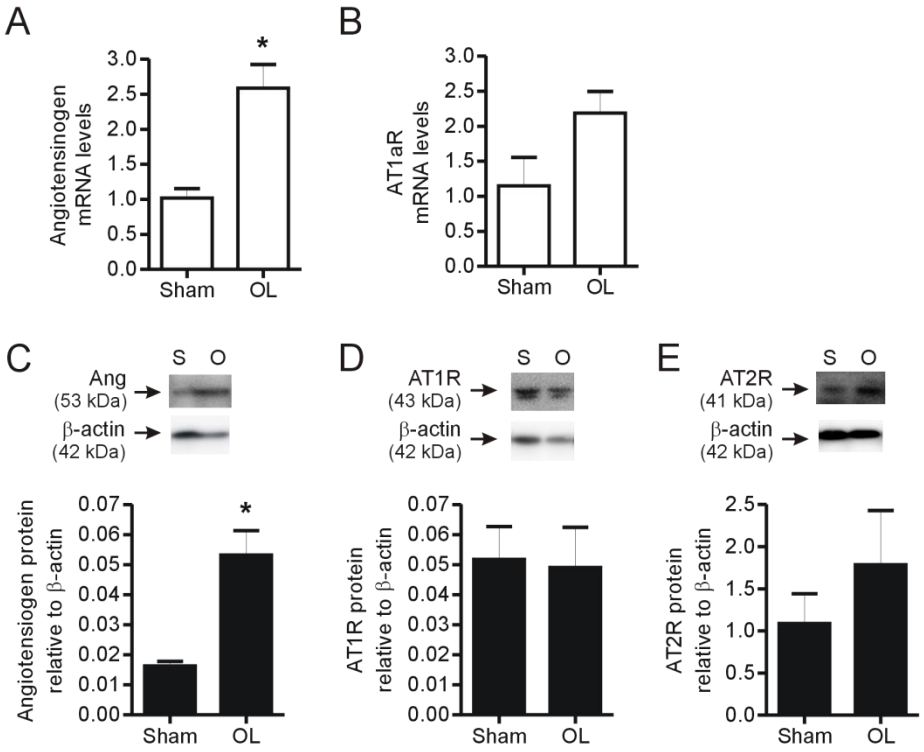


Figure A-4: Effect of skeletal muscle overload on angiotensinogen and AT receptor expression in skeletal muscle.
Muscle subjected to overload for 5 days was lysed for qPCR analysis of angiotensinogen (A) and AT1aR (B) transcript levels. Protein levels of Angiotensinogen (C), AT1R (D) and AT2R (E) were assessed by Western Blotting. In each graph, values are presented as mean ± SEM (n=3 for Sham and n=6 for Overload). Student's t-test (*) revealed a significant difference between angiotensinogen transcript and protein levels in overload compared to sham animals. OL overload.

Gorman, J. L., Liu, S. T. K., Slopack, D., Shariati, K., Hasanee, A., Olenich, S., Olfert, M., Haas, T. L. (2013). Angiotensin II evokes angiogenic signals within skeletal muscle through co-ordinated effects on skeletal myocytes and endothelial cells. *PLoS One.* 2014 Jan 9;9(1):e85537.

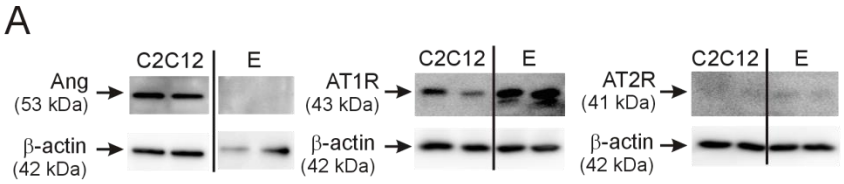


Figure A-5: Contribution of endogenous Ang II to muscle VEGF production and endothelial cell-muscle cell crosstalk.
C2C12 myotubes and confluent cultures of microvascular endothelial cells were lysed, and endogenous basal levels of angiotensinogen, AT1R and AT2R were assessed by Western blot analysis (A). In all blots, two independent cultures are shown for each cell type. E endothelial cell.

Gorman, J. L., Liu, S. T. K., Slopack, D., Shariati, K., Hasanee, A., Olenich, S., Olfert, M., Haas, T. L. (2013). Angiotensin II evokes angiogenic signals within skeletal muscle through co-ordinated effects on skeletal myocytes and endothelial cells. *PLoS One*. 2014 Jan 9;9(1):e85537.

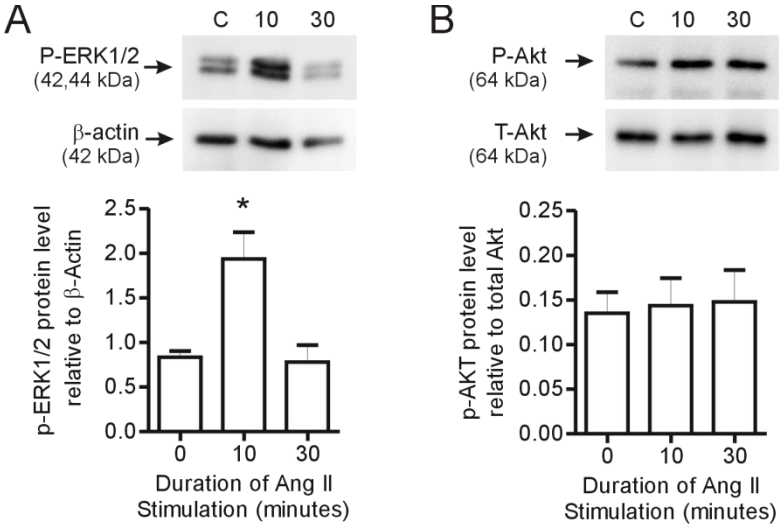


Figure A-6: Effect of Ang II stimulation on VEGF and MMP-2 expression in C2C12 myotubes.
C2C12 myoblasts were differentiated in 2% serum media for 5-8 days until myotubes had formed. C2C12 myoblasts were stimulated for either 10 or 30 minutes with 1 mM Ang II and then lysed for protein analysis. P-ERK1/2 (n=3 per condition) (A) and P-Akt (n=5 per condition) (B) levels were assessed by Western blotting with activated levels normalized to b-actin and total Akt levels respectively. Values are presented as mean ± SEM. Student's t-test and one way ANOVA followed by Bonferroni's multiple comparison test were used to assess statistical significance which was set as $p<0.05$. In panel A * denotes $p<0.05$ vs. untreated cells. C Control, 10 10 minutes and 30 30 minutes.

Gorman, J. L., Liu, S. T. K., Slopack, D., Shariati, K., Hasanee, A., Olenich, S., Olfert, M., Haas, T. L. (2013). Angiotensin II evokes angiogenic signals within skeletal muscle through co-ordinated effects on skeletal myocytes and endothelial cells. *PLoS One.* 2014 Jan 9;9(1):e85537.

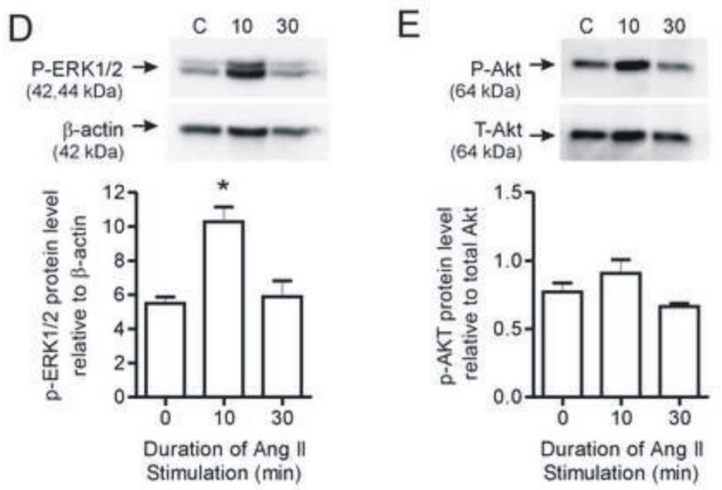


Figure A-7: Effect of Ang II stimulation on VEGF and MMP-2 expression in skeletal muscle endothelial cells.

Endothelial cells were stimulated for either 10 or 30 minutes with 1 mM Ang II and then lysed for protein analysis. P-ERK1/2 (D) and P-Akt (E) levels were assessed by Western blotting and normalized to b-actin and total Akt levels respectively (n=3 per condition). Values are presented as mean ± SEM. One way ANOVA followed by Tukey's multiple comparison test and student's t-test were used to assess statistical significance ($p<0.05$). In panel D * denotes $p<0.05$ vs. untreated cells. C Control, 10 10 minutes and 30 30 minutes.

Gorman, J. L., Liu, S. T. K., Slopack, D., Shariati, K., Hasanee, A., Olenich, S., Olfert, M., Haas, T. L. (2013). Angiotensin II evokes angiogenic signals within skeletal muscle through co-ordinated effects on skeletal myocytes and endothelial cells. *PLoS One.* 2014 Jan 9;9(1):e85537.

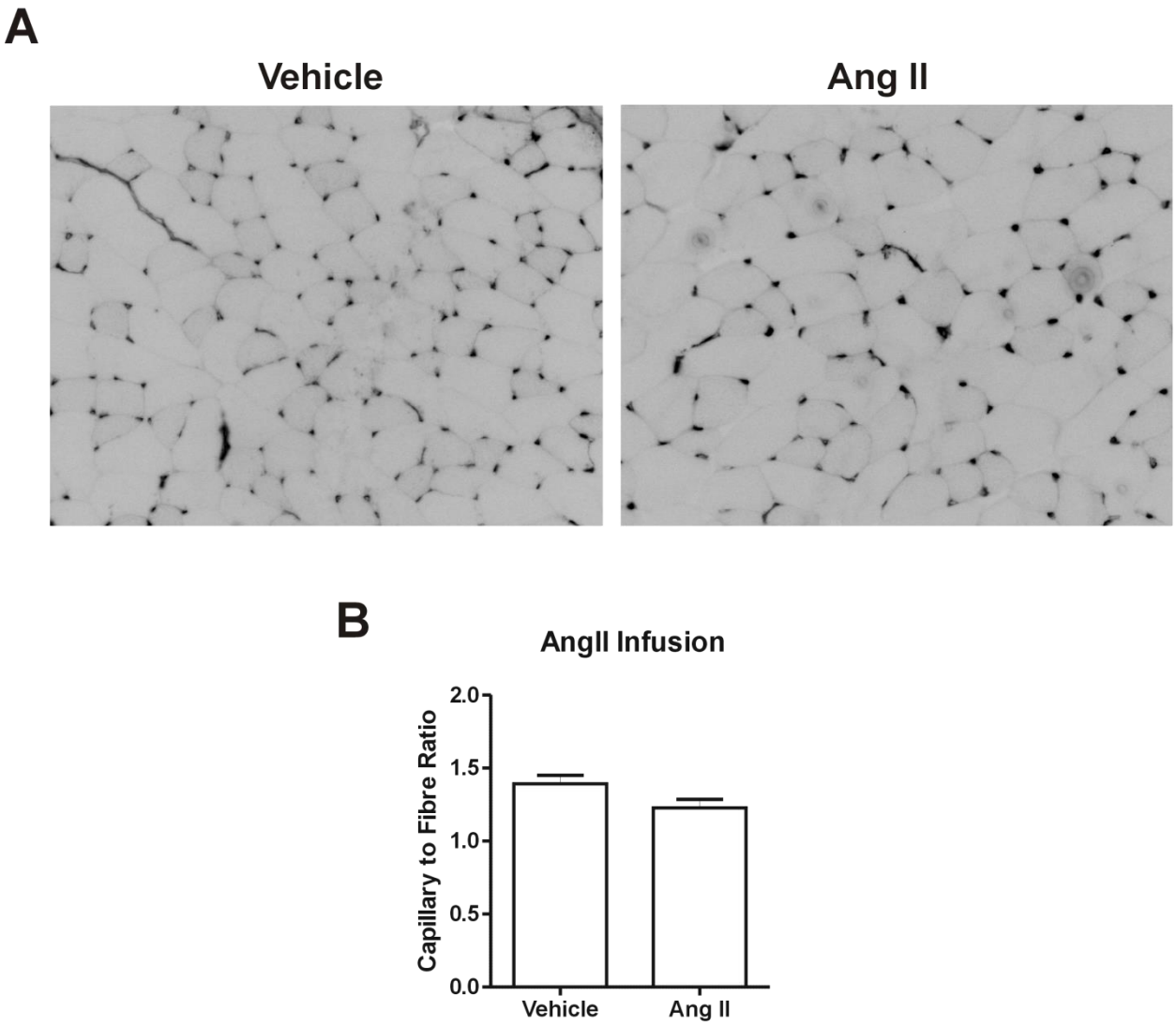


Figure A-8: Exogenous Ang II infusion did not alter capillary growth.
Representative inverted grey-scale images of iso-lectin staining (A) were used to calculate the capillary-to-muscle fibre ratio (n=5 for Vehicle, n=5 for Ang II) (B). Values are presented as mean ± SEM. Student's t-test revealed no significant difference in capillary-to-fibre ratio between vehicle and Ang II infusion.

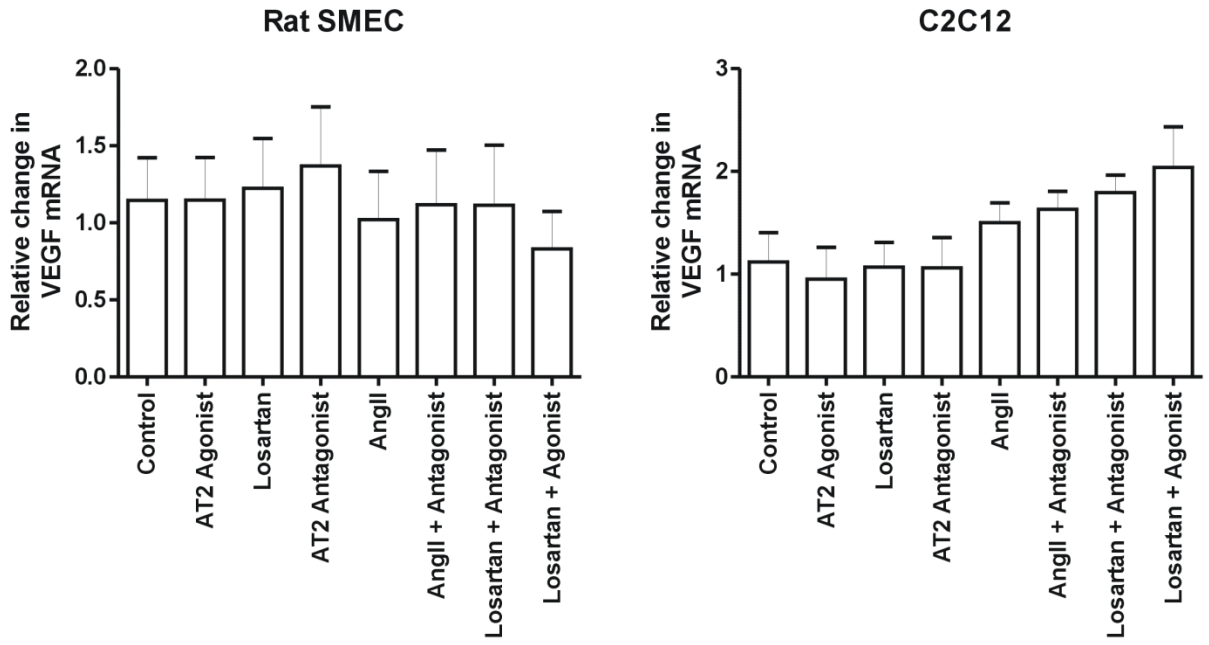


Figure A-9: Ang II, losartan, AT2R agonist, and AT2R antagonist treatments did not change VEGF expression in skeletal muscle endothelial cells and C2C12 myoblast.
Culture rat skeletal muscle endothelial cells (A) and C2C12 myoblast (B) were treated with 100nM of Ang II, 1mM of losartan, 1mM of AT2R antagonist, and 100nM of AT2R agonist overnight. Cells were lysed for qPCR analysis of VEGF transcript levels (n=4 per group). Values are presented as mean ± SEM. There were no significant differences between treatment groups in both cell types, as assessed by one way ANOVA.

FoxO1 and FoxO3a are involved in the regulation of exercise induced angiogenesis
(Slopack et al., manuscript in progress)

Study objective

FoxO1 and FoxO3a are expressed in endothelial cells and their expression is known to have anti-angiogenic effects (Tatsuo Furuyama et al., 2004; Paik et al., 2007). The involvement of FoxO transcription factors in the regulation of exercise induced capillary growth is not known. The objective of this study is to assess whether FoxO1 and FoxO3a are regulated in endothelial cells in response to aerobic exercise, and in turn, contribute to the angiogenic response seen with training.

Results

FoxO3a protein was elevated after a single bout of treadmill running and returned to resting levels with 5 days of training. FoxO1 and FoxO3a levels were significantly increased after 28 days of training, a time point where there is no further increase in capillary-to-fibre ratio compared to 14 days of exercise. Mice with endothelial cell directed deletion of FoxO1/3/4 have reduced vascular endothelial expression of FoxO1 and FoxO3a, reduced expression of thrombospondin1 and displayed an accelerated angiogenesis response to repeated bouts of endurance exercise. Both FoxO1 and FoxO3a play a role in mediating the angiogenic response elicited by aerobic exercise.

Project contribution

My involvement with this project includes monitoring the mice exercise training regimen on the treadmill, endothelial cell isolation from mouse skeletal muscle, and

assisted with skeletal muscle extraction from mice following exercise. I also performed mRNA analysis on FoxO1 (Figure A-10 A, Figure A-11 A), FoxO3a (Figure A-10 B, Figure A-11 b), TSP-1 (Figure A-12), and Gadd45 (Figure A-13) in response to various exercise training time points.

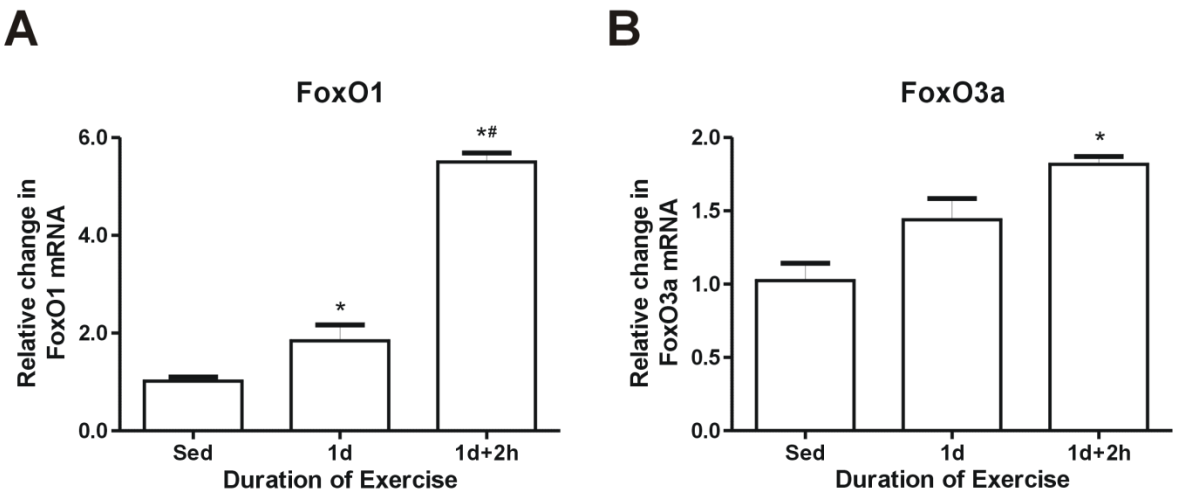


Figure A-10: FoxO1 and FoxO3a expression were elevated with acute exercise.
Mice trained for 1 day (1d) or remained sedentary (Sed). Muscles were extracted immediately after (1d) or 2 hours after (1d+2h) exercise. (A) FoxO1 (n=5 for sed, n=4 for 1d and 1d+2h) and (B) FoxO3a (n=5 for sed, n=4 for 1d and 1d+2h) transcript levels were analyzed by qPCR. Values are presented as mean ± SEM. Significant training effect ($p<0.05$) (one way ANOVA) *=$p<0.05$ vs. Sedentary, #=$p<0.05$ vs. 1 day.

Slopack, D., Liu, S.T.K., Roudier, E., Birot, O., Haas, T.L., Forkhead BoxO transcription factors restrain exercise-induced angiogenesis (manuscript in progress)

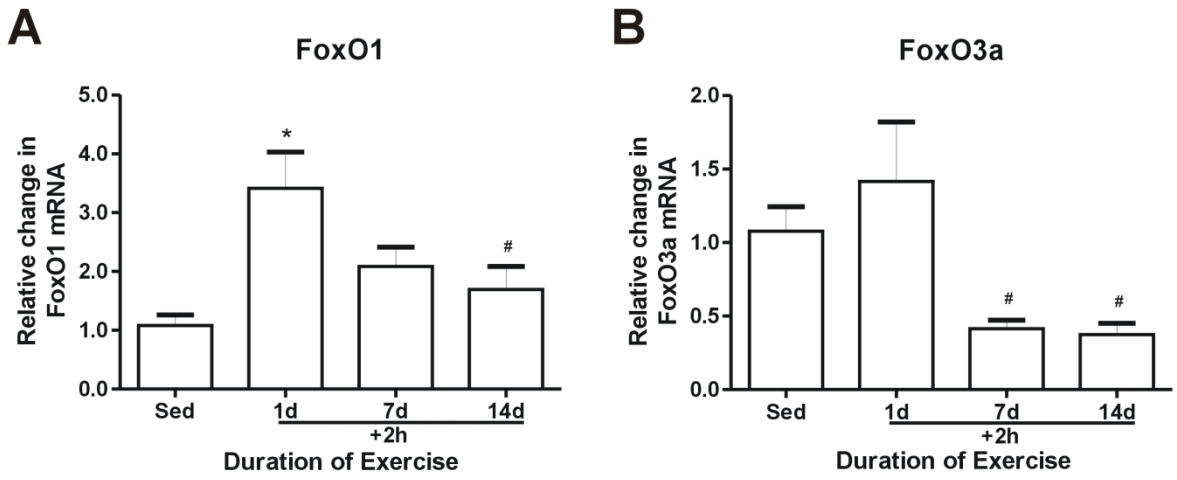


Figure A-11: Exercise training elicited temporal changes in FoxO1 and FoxO3a expression. Mice trained for 1, 7, 14 day (1d, 7d, 14d) or remained sedentary (Sed). Muscles were extracted 2 hours after training. (A) FoxO1 (n=6 per group) and (B) FoxO3a (n=6 per group) transcript levels were analyzed by qPCR. Values are presented as mean ± SEM. Significant training effect ($p<0.05$) (one way ANOVA) *=$p<0.05$ vs. Sedentary, #=$p<0.05$ vs. 1d day.

Slopack, D., Liu, S.T.K., Roudier, E., Birot, O., Haas, T.L., Forkhead BoxO transcription factors restrain exercise-induced angiogenesis (manuscript in progress)

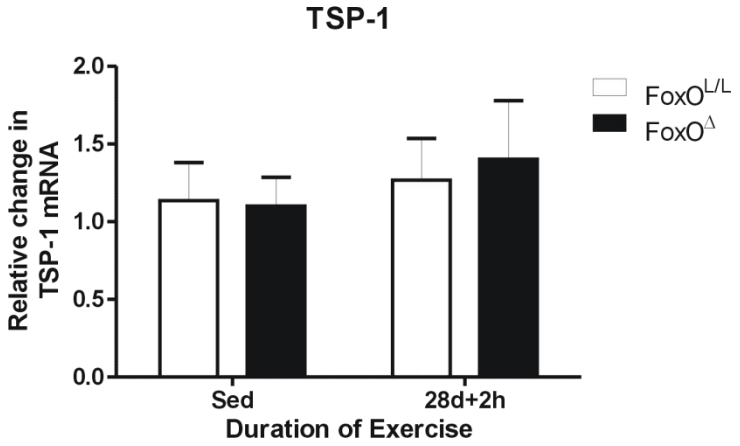


Figure A-12: TSP-1 expression was not altered with chronic training in the FoxO$^{\Delta}$ model.
Mice trained for 28 day (28d) or remained sedentary (Sed). Muscles were extracted 2 hours after training. TSP-1 transcript levels were analyzed by qPCR. Values are presented as mean ± SEM. There was no significant difference between genotypes and training, as assessed by two way ANOVA. (n=6 for Sed and n=5 for 28d+2h in FoxO$^{L/L}$, n=6 for Sed and n=7 for 28d+2 in FoxO$^{\Delta}$).

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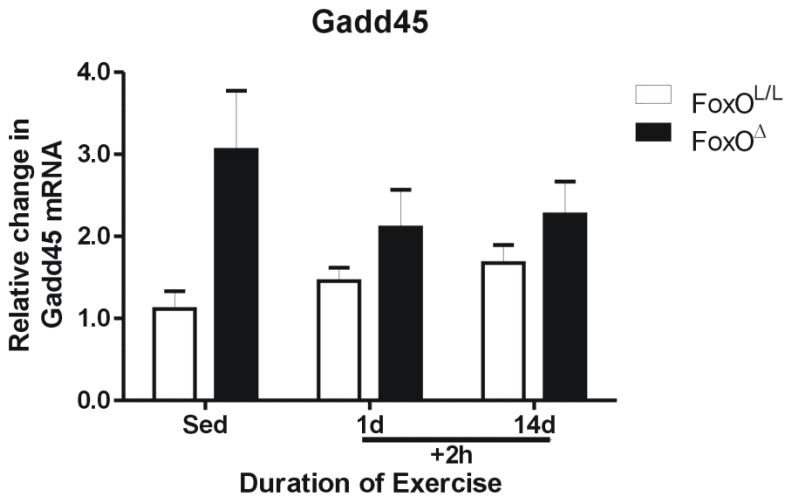

Figure A-13: Gadd45 expression did not change with exercise training in the FoxO$^{\Delta}$ model.
Mice trained for 1, 14 day (1d, 14d) or remained sedentary (Sed). Muscles were extracted 2 hours after training. Gadd45 transcript levels were analyzed by qPCR. Values are presented as mean ± SEM. There was no significant difference between genotypes and training, as assessed by two way ANOVA. (n=6 per group in FoxO$^{L/L}$, n=6 for per group in FoxO$^{\Delta}$).

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