

**Investigating Murine Double Minute-2 pro-angiogenic function in  
health and disease: an integrative approach from rodent and human  
skeletal muscle to primary endothelial cells**

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## Abstract

Capillaries are essential for overall skeletal muscle health, transporting nutrients and oxygen as well as removing waste from muscle cells. Angiogenesis is the process of capillary growth from pre-existing capillaries, and is regulated by a balance of pro- and anti-angiogenic factors. Our laboratory has demonstrated that Murine Double Minute-2 (Mdm2) is essential for skeletal muscle capillary maintenance as well as for exercise-induced angiogenesis. The aim of my research was to further investigate Mdm2 function in both physiological (exercise) and pathological (diabetes) contexts at the tissue level of the skeletal muscle and in primary endothelial cells. I first contributed to show that Mdm2 protein levels are elevated following a prolonged exercise training program in human skeletal muscle (*academic research paper 1*). Next, I investigated the response of Mdm2 to an acute bout of exercise, which itself represents a powerful pro-angiogenic stimulus (*academic research paper 2*). Muscle contractile activity stimulates Mdm2 phosphorylation on its serine 166 concomitant with increased pro-angiogenic vascular endothelial growth factor-A (VEGF-A). Mdm2 phosphorylation was found to be dependent on VEGF-A signaling, demonstrating for the first time that Mdm2 and VEGF-A can interact in a complex regulatory loop. *In vitro* experiments show that VEGF-A-dependent activation of Mdm2 leads to increased migratory activity of endothelial cells. This effect appeared to be a result of enhanced Mdm2-FoxO1 binding, resulting in the inhibition of FoxO1-dependent regulation of thrombospondin-1 expression, a potent anti-angiogenic molecule. I further explored this relationship between VEGF-A and Mdm2 in *academic research paper 3*, demonstrating that VEGF-driven Mdm2 phosphorylation was dependent on extracellular signal related kinases 1/2 (ERK1/2) - p90 ribosomal s6 kinase (p90RSK) in primary human endothelial cells. Finally, *academic research paper 4* provided evidence that Mdm2 is a clinically relevant protein in type 1 diabetes (T1D), a highly understudied disease in the context of

skeletal muscle angio-regulation. Mdm2 protein abundance was lowered in muscles from T1D animals alongside increases in key anti-angiogenic Mdm2 targets and significant capillary regression. Taken together, my dissertation research clearly identifies Mdm2 as a master regulator of angio-adaptation at the level of the skeletal muscle and endothelial cells in both health and disease.

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## List of abbreviations

AhR	Aryl hydrocarbon receptor
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
Akt	Protein kinase-B
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
BB Rats	BioBreeding type 1 diabetic rats (either diabetes prone or resistant)
Egr-1	Early growth response protein 1
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal related kinases 1/2
ERR $\alpha$	Estrogen related receptor alpha
FGF-2	Fibroblast growth factor-2
FoxO1	Forkhead box protein O1
HAMEC	Human adipose microvascular endothelial cells
HDMEC	Human dermal microvascular endothelial cells
HIF-1 $\alpha$	Hypoxia inducible factor 1-alpha
HIF-1 $\beta$	Hypoxia inducible factor 1-beta
HRE	Hypoxia responsive element
IP	Immunoprecipitation
MAPK	Mitogen activated protein kinases
Mdm2	Murine double minute-2

Mdm2 <sup>Puro/<math>\Delta</math>7-9</sup>	Mdm2 deficient mice
mSMEC	Mouse skeletal muscle endothelial cells
NO	Nitric oxide
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
p90RSK	p90 ribosomal s6 kinase
PDGF-B	Platelet derived growth factor-B
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositide 3-kinase
p-Ser166-Mdm2	Mdm2 phosphorylated on serine 166
recVEGF-A	Recombinant VEGF-A protein
S166D-Mdm2	Ser166-Mdm2 phosphorylation mimetic–overexpressing endothelial cells
SIRT-1	Sirtuin-1
Sp1	Specificity protein 1
SSREs	Shear stress responsive elements
TGF- $\beta$	Transforming growth factor- $\beta$
TSP-1	Thrombospondin-1
USFs	Upstream stimulatory factors
VEGF-A	Vascular endothelial growth factor-A
VEGFR2	Vascular endothelial growth factor receptor 2
ZDF	Zucker diabetic fatty rats
WT-Mdm2	Wild-type Mdm2-overexpressing endothelial cells

# 1. INTRODUCTION & LITERATURE REVIEW

## 1. The skeletal muscle microvasculature

### 1.1 *The importance of the skeletal muscle microvasculature*

The skeletal muscle tissue accounts for 40% of our body weight and is essential for a variety of functions including metabolic regulation and locomotion [1]. The basal metabolism of the skeletal muscle tissue requires roughly 20% of total cardiac output and with acute exercise blood flow to the muscle tissue can increase by 20 fold or more during maximal exercise [2-5]. In order to ensure proper functioning, active muscle cells must be supplied with adequate oxygen and nutrient delivery along with the removal of metabolic wastes. The vascular system is composed of blood vessels ranging in size from large arteries and veins and smaller arterioles and venules (macrovasculature) to the capillaries (microvasculature). Capillaries are our smallest blood vessels with an average diameter of around 5 $\mu$ m [6,7]. The capillary network serves as the site of gas and nutrient exchange in all bodily tissues including the skeletal muscle [8,9]. In the skeletal muscle, capillaries are intimately associated with muscle fibers, running parallel and longitudinally to the fibers, with short lateral branches forming vascular networks. In 1919, a pioneering theory was put forth by August Krogh that describes each capillary as supplying a cylindrical area of muscle with oxygen and nutrients which travel from the bloodstream, out of the capillary, and to the muscle cells [10]. As oxygen has a limited diffusion distance, the density and distribution of capillaries surrounding muscle fibers determines the oxygenation and the metabolic capacity of the muscle tissue [11]. The oxygen capacity of the skeletal muscle is largely predicted by the capillarization of the tissue and is a direct determinant of exercise capacity, since exercise requires large increases in blood flow to working muscle groups [2]. Indeed, it is well documented that more oxidative

muscle types have a greater capillary density [12]. Since each individual capillary has the capacity to supply only a limited surface area of muscle, we can appreciate that the loss of capillaries can likely result in loss of muscle function. All blood vessels, including our capillaries, are made up of endothelial cells and, as such, it is the regulation and activity of the endothelial cell itself that largely determines overall microvascular function.

## **1.2** *Skeletal muscle capillary plasticity*

The skeletal muscle capillary network shows remarkable plasticity, adapting to both physiological and pathological conditions by capillary expansion or regression. In healthy adults, endothelial cells are usually quiescent, and do not divide, with cell turnover time in the hundreds of days [13]. However, in response to pathological stimuli such as tumor growth and wound repair, or physiological stimuli such as menstruation and exercise training, endothelial cells begin to proliferate and migrate to form new capillaries [2].

### **1.2a** *Vasculogenesis*

The *de novo* formation of the vascular network is termed vasculogenesis, and is one of the first steps in embryo development [14]. This process entails the aggregation of hemangioblasts, which are blood and endothelial precursor cells, forming the first vascular structures termed blood islands [15]. The formation of a primary capillary plexus is achieved through the fusion of multiple blood islands [15]. Maturation of this primary plexus involves cell proliferation, migration and angiogenesis, which is discussed in subsequent sections. Interestingly, vasculogenesis is not restricted to the developing embryo, as it also can occur in adults in response to acute and chronic ischemic conditions that result in hypoxia such as vascular disease, wound healing, tumor growth, retinopathy, and during exercise [16]. The process whereby endothelial precursor cells lead to

adult neovascularization is termed postnatal vasculogenesis, and is driven by bone-marrow derived endothelial progenitor cells (EPCs) [17,18]. An elevation in circulating EPCs that are released from the bone marrow travel to ischemic, hypoxic, or injured tissue and differentiate into mature endothelial cells [18]. Indeed, a number of studies demonstrate that exercise-induced hypoxia and muscle ischemia increase the amount of circulating EPCs and promote neovascularization [16,19-22].

### **1.2b Arteriogenesis**

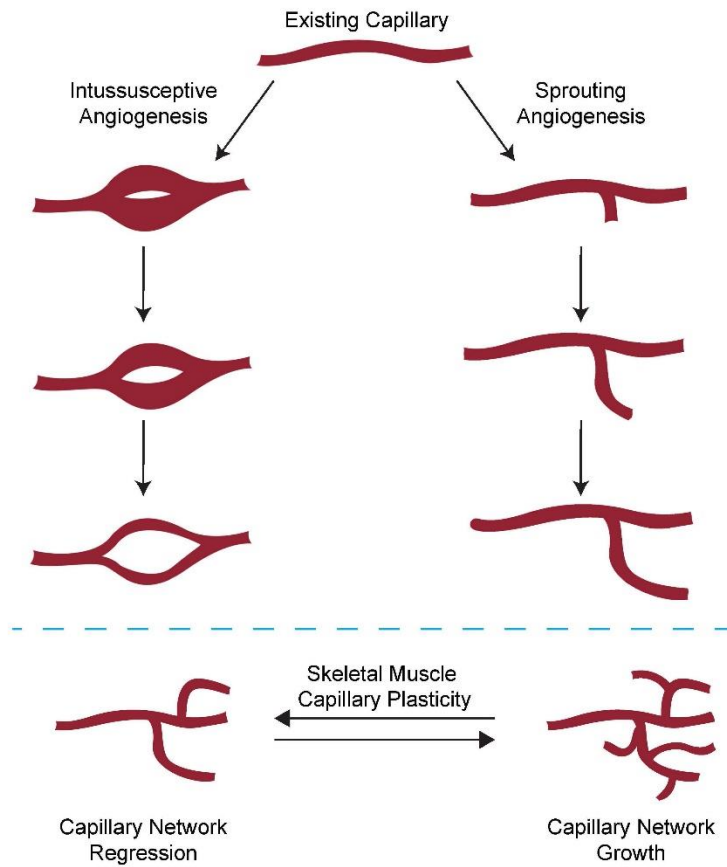
Arteriogenesis is the process through which existing arteries and arterioles are enlarged in both diameter and wall thickness leading to an overall larger blood vessel involving the remodelling of endothelial cells, vascular smooth muscle cells (VSMC), and fibroblasts [2,23]. While older literature also refers to arterial collateralization and capillary arterialization as arteriogenesis, these are separate processes whereby new arterial conduit vessels are formed to bypass an occlusion, or existing mature capillaries are surrounded by vascular smooth muscle cells, respectively [2]. The purpose of arteriogenesis is to increase the blood flow capacity of a resistance vessel (a vessel that has the ability to dilate or constrict in order to regulate blood flow to downstream tissues). Since arteriogenesis cannot compromise the vessel's ability to dilate and constrict, thickening of the vascular wall (due to VSMC and extracellular matrix remodelling) must also occur in addition to increases in diameter [2]. The velocity of blood flow, elevated internal pressure, and the total volume of blood a resistance vessel experiences directly dictates the size of that vessel, as this exposes the endothelium to greater shear stress (the physical drag endothelial cells experiences as blood rushes past in the vessel lumen [23]). Prolonged exercise training can induce arteriogenesis by intermittently increasing the shear stress experienced by the arterial endothelium during exercise bouts as exercise increases heart rate, stroke volume and thus

cardiac output. Indeed, it has been demonstrated that the size of major vessels is tightly associated with the level of exercise training or disuse of a specific downstream muscle group [24].

### **1.2c** *Angiogenesis*

The process whereby new capillaries are formed from pre-existing ones is termed angiogenesis. Angiogenesis is essential for a number of physiological processes including organ and tissue regeneration and repair, during wound healing, menstruation and pregnancy, and exercise training [25-28]. There are two distinct processes in which new capillaries are formed from pre-existing ones, termed ‘sprouting’ and ‘intussusceptive’ (splitting) angiogenesis, respectively. Sprouting angiogenesis first involves the activation of endothelial cells, a degradation of the basement membrane and extracellular matrix (the structural area surrounding the capillary), endothelial cell proliferation, migration and subsequent lumen formation [29-31]. The second phase sees the new vessel mature and differentiate through the recruitment of associated cell types such as pericytes, the addition of extracellular matrix around the sprout, and re-entry into the capillary bed to form a capillary loop [29-31]. Sprouting angiogenesis is observed in response to muscle activity as is seen during chronic electrical stimulation [32]. Alternatively, intussusceptive angiogenesis involves the insertion of endothelial cell projections, or pillars, within the lumen of the vessel eventually splitting the original lumen into new vessels [33,34], and occurs in response to elevated blood flow [7]. Splitting angiogenesis is understood as being a more energy efficient form of new capillary formation, as it requires less endothelial cell proliferation and does not require basement membrane degradation to occur [23]. Refer to figure 1.1 for a schematic overview of sprouting versus intussusceptive angiogenesis. Increased capillary branching results in greater surface area and increased capillary-muscle interface [2]. This adaptation allows for

increased blood residence time and therefore increased oxygen and substrate exchange time in combination with decreased diffusion lengths [2].



**Figure 1.1** The difference between sprouting and intussusceptive angiogenesis. Sprouting angiogenesis involves the proliferation and migration of endothelial cells to form a vascular sprout leading to a new capillary. Intussusceptive angiogenesis occurs when tissue pillars project into an existing lumen to form new, separate lumens. Both sprouting and intussusceptive angiogenesis leads to increased branching of the capillary network, while capillary regression, or pruning, leads to fewer capillaries.



### **1.3 *Measurements of skeletal muscle angiogenesis / capillary regression***

The capillary density (CD) is commonly measured to determine the extent of skeletal muscle perfusion, and is given by the cross-sectional counting of the total number of capillaries in a given surface area of muscle. Occasionally, the CD is wrongfully used as a true indicator of angiogenesis. Since the CD can increase or decrease as a result of muscle atrophy or hypertrophy, respectively, with no actual capillary growth or regression occurring, it cannot be used as a measurement of true angiogenesis. Instead, the CD could be considered a way to appreciate blood perfusion to the muscle, as it reflects the amount of capillaries present in a given surface area of muscle tissue. A true indication of skeletal muscle angiogenesis is the capillary-to-fiber (C/F) ratio, which is given by taking the ratio of capillaries to muscle fibers in a cross-sectional area of a muscle biopsy. The C/F ratio is not affected by muscle atrophy or hypertrophy, and therefore is a reliable measurement to assess skeletal muscle capillarization and angiogenesis.

### **1.4 *Exercise-induced skeletal muscle angiogenesis***

The process of skeletal muscle angiogenesis in response to exercise training, termed exercise-induced angiogenesis, is a main focus of this dissertation. It is well documented that exercise training leads to improved blood flow capacity to working muscle groups [35]. Several mechanisms contribute to establish this exercise-induced adaptation (i.e. increased cardiac output and arteriogenesis), with muscle capillarity being an important determinant of the overall blood flow capacity of the skeletal muscle. As previously stated, the capillary network matches oxygen and nutrient delivery to the metabolic needs of muscle cells. Thus, in response to repetitive muscle contractile activity, the capillary network must adapt to the increase in metabolic demand of active muscle cells [1]. Angiogenesis, therefore, plays a critical role in muscle adaptation to exercise training [2]. In humans, endurance exercise training is a strong driving force for skeletal muscle

angiogenesis, with training programs lasting between 4 to 24 weeks in duration leading to 10-25% increased muscle capillarity in most cases [36]. Indeed, it is well documented that aerobic endurance exercise leads to skeletal muscle angiogenesis in response to numerous exercise modalities [1]. Aerobic exercise may induce increased muscle capillarization independent of changes in fiber size, however the cross-sectional area of muscle fibers typically remains unaltered and the changes in capillary density and capillary-to-fiber ratio are unidirectional [36]. In contrast, heavy resistance exercise in which skeletal myocytes undergo hypertrophy can lead to decreases in capillary density, due to increased muscle cross-sectional area [37]. Resistance programs that incorporate higher repetition exercises may prevent such a loss in capillary density [2,29]. Indeed, several studies show an increase in the capillary-to-fiber ratio in response to resistance exercise training, preserving the capillary density and therefore the perfusion of the muscle [38-40]. Furthermore, a study from Gavin and colleagues [41] shows that the response of angio-adaptive growth factors are similar in magnitude and timing in response to resistance versus aerobic exercise.

Exercise can promote skeletal muscle angiogenesis through several specific mechanisms that provide stimulus for the growth of skeletal muscle capillaries. The physical environment of the microvasculature is an important determinant of whether the capillary network is maintained, expands, or regresses. Physical forces are always present at the level of the endothelium, and all blood vessels are exposed to mechanical stimuli from both the surrounding area such as stretch and muscle contraction and from intravascular stresses such as blood pressure and shear stress [42]. All of these mechanical forces have the ability to contribute to the formation of new capillaries in response to exercise.

As stated previously, shear stress is a mechanical stress defined as the physical drag endothelial cells experience as blood rushes past in the vessel lumen [23]. Unlike arterioles, capillaries lack the ability to regulate their diameter due to the absence of vascular smooth muscle cells surrounding the vessel. Therefore, the adaptation of the capillary network to shear stress differs to the process of arteriogenesis. Instead of an enlargement of the vessel, the capillary network can expand by angiogenesis to allow a greater amount of blood to flow through the muscle tissue due to increased capillary surface area, mainly through intussusceptive vs. sprouting angiogenesis [42,43]. Shear stress is sensed by endothelial cells by mechanotransducers present on the cell surface such as G-proteins, cell-matrix and cell-cell adhesion molecules, ion channels, and tyrosine kinases (such as the vascular endothelial growth factor receptor 2 [VEGFR2]) [36,44,45]. When mechanoreceptors sense shear stress they become activated and initiate downstream signaling through mitogen activated protein kinases (MAPKs) and phosphatidylinositide 3-kinase (PI3K)-Akt signaling [44,45]. Shear stress induces alterations in gene expression by increasing the activity of a number of transcription factors including early growth response protein 1 (Egr-1), activator protein-1 (AP-1), specificity protein 1 (Sp1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) [44,46] that bind to common promoter regions in target genes termed shear stress responsive elements (SSREs) [44,47] with subsequent expression of growth factors and molecules involved in angiogenesis such as endothelial nitric oxide synthase (eNOS), nitric oxide (NO), VEGFR2, vascular endothelial growth factor-A (VEGF-A; see following section discussing VEGF-A), transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet derived growth factor-B (PDGF-B), and fibroblast growth factor-2 (FGF-2) [2,43,44,46,48-50].

Skeletal muscle endothelial cells are also exposed to additional mechanical forces not present in other tissues due to the contractile properties of muscle fibers. Zhou et al [51] demonstrated in rodents that extirpating the tibialis anterior resulting in increased stretch and overload of the synergist muscle, extensor digitorum longus, led to capillary growth by sprouting in the overloaded muscle. Indeed, muscle stretch leads to sprouting angiogenesis whereas shear stress favors splitting angiogenesis [42,43]. Both increased blood flow [52,53] and increased muscle stretch [54] have been shown in human models of passive exercise (i.e. no active work being performed) through motor-driven passive flexion and extension on the leg. Passive exercise has been shown to increase pro-angiogenic factor expression [52], and while 4 weeks of passive exercise did not result in overt angiogenesis, a higher proportion of proliferating endothelial cells were detected [54], suggesting that passive stretch has the ability to initiate the angiogenic process. The passive stretch model requires minimal metabolic demand, therefore isolating and highlighting the importance of purely mechanical forces to the angiogenic process in the skeletal muscle.

In addition to mechanical forces, the skeletal muscle may experience exercise-induced local hypoxia, which could serve a strong pro-angiogenic stimulus. Reduced oxygen levels available to the muscle tissue is a result of elevated oxygen consumption in the active muscle compared to resting [55]. The O<sub>2</sub> cascade is defined as the transport of oxygen from the air we breathe to the utilization of oxygen in the mitochondria. This transport system involves the lungs, heart, vascular circulation, and the muscle tissue [56]. As oxygen is transported along this anatomical pathway, the partial pressure of oxygen (PO<sub>2</sub>) steadily declines. The PO<sub>2</sub> of the air we breathe is approximately 149 mmHg, yet falls to 105 mmHg in lung alveoli, 103 mmHg in arterial circulation, 44 mmHg on average at the level of the capillaries, and 34 mmHg inside muscle cells

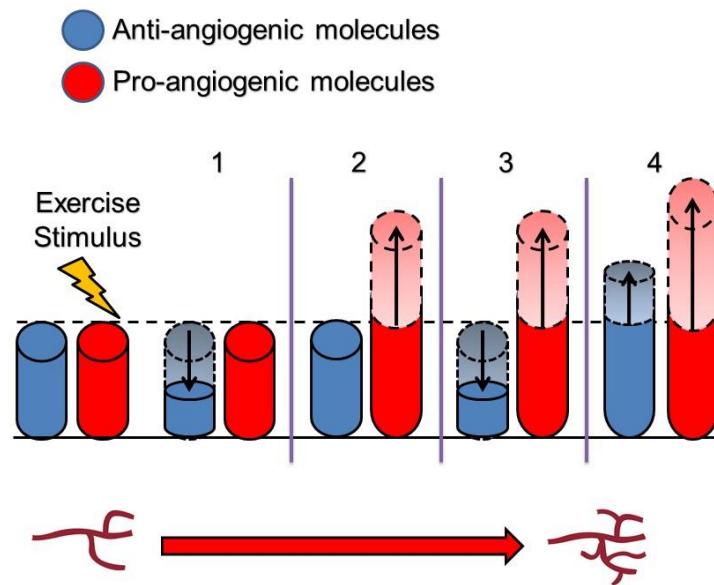
in humans [57]. A bout of exercise strongly alters  $PO_2$  at every point along this pathway, and results in comparative hypoxia at the level of the myocyte versus resting conditions. In response to maximal knee extensor exercise, alveolar (120 mmHg) and arterial (115 mmHg)  $PO_2$  are elevated due to increased respiration, however, the mean capillary  $PO_2$  (37.5 mmHg) and intramyocyte  $PO_2$  (3.1 mmHg) are reduced [57,58]. It can be appreciated that endothelial cells at the level of the capillary bed perhaps experience mild hypoxia during exercise (44 mmHg resting versus 37.5 mmHg during exercise), however the drop in  $PO_2$  at the level of the myocyte is profound (34 mmHg resting versus 3.1 mmHg during exercise), representing a dramatic hypoxic state compared to rest. Correspondingly, it has been reported that myoglobin oxygen saturation is shifted from ~100% saturation at rest to ~50% saturation during exercise in the human leg [56,58]. This alteration in myoglobin saturation was observed as rapidly as 30 seconds after the onset of exercise, and returns to resting values in less than 1 minute of exercise cessation [56,58].

The cellular response to hypoxia is largely sensed by the transcription factor hypoxia inducible factor-1 (HIF-1) which consists of two subunits, the oxygen sensitive HIF-1 $\alpha$  (which is stabilized by low cellular oxygen levels, and rapidly degraded in normoxia) and the constitutively active HIF-1 $\beta$  [55]. In the hypoxic state, HIF-1 $\alpha$  is stabilized and shuttles from the cytoplasm to the nucleus where it heterodimerizes with the  $\beta$  subunit subsequently interacting with hypoxia responsive elements (HRE) in target genes that promote increases in oxygen transport such as VEGF-A (stimulates angiogenesis) and erythropoietin (EPO; stimulates erythropoiesis), while concomitantly upregulating glucose receptors and glycolytic enzymes [55,59,60]. Indeed, Jiang et al [61] show that HIF-1 $\alpha$  protein accumulation is attributable to oxygen levels, and that lower oxygen levels stimulated increased HIF-1 $\alpha$ -DNA binding in HeLa cells. Localized muscle hypoxia is a pro-angiogenic stimulus, as stabilization of HIF-1 $\alpha$  in a specific area of muscle tissue can lead

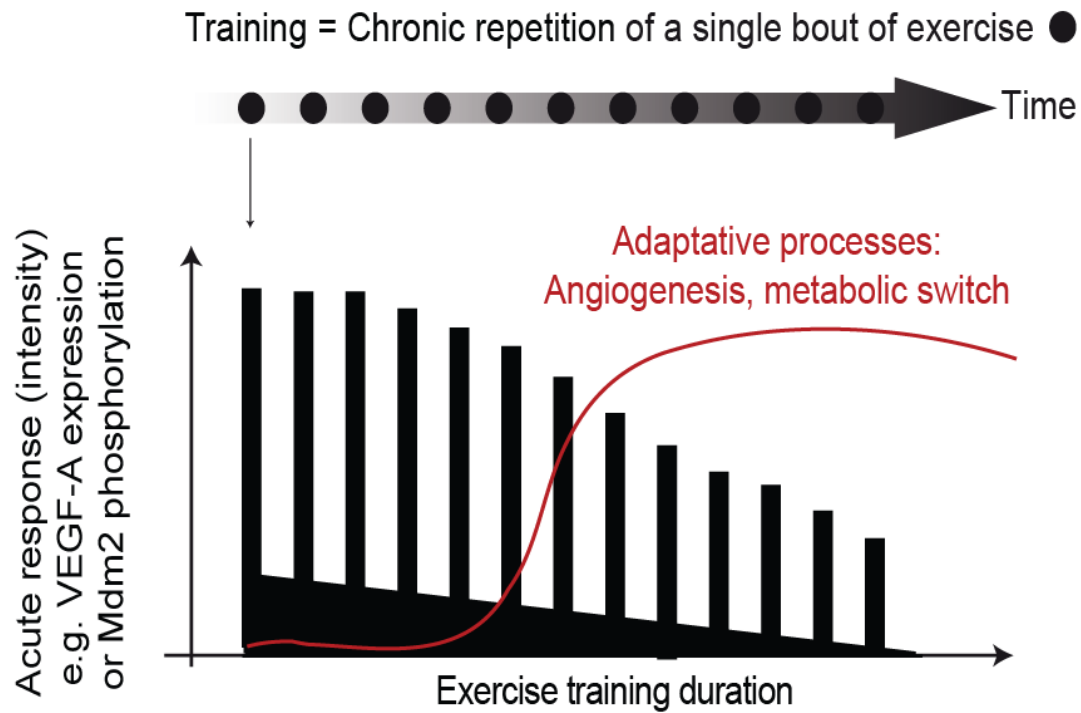
to increased myocyte expression and release of the pro-angiogenic VEGF-A which can serve as a chemoattractant toward which endothelial cells from existing vessels can migrate in order to form new capillaries. Indeed, it has been documented that exercise increases HIF-1 $\alpha$  protein immediately following and up to 6 hours post-exercise in human skeletal muscle, corresponding to increased VEGF-A and EPO mRNA levels [62]. As the adaptation of the muscle to exercise is established (i.e. exercise-induced angiogenesis), the response of HIF-1 $\alpha$  is attenuated, as the observed increase in HIF-1 $\alpha$  expression in response to an acute bout of exercise was lost following 4 weeks of training in human skeletal muscle [63]. Interestingly, it has been shown that HIF-1 $\alpha$  regulation is possible aside from hypoxic stress by insulin and insulin-like growth factors, platelet-derived growth factor, and epidermal growth factor as well as the activation of the signaling cascades PI3K/Akt and RAS/ERK1/2 [59,60].

Often, when exercise-induced angiogenesis is discussed, we are referring to the outcome, or the end result, following a period of exercise training when an increased abundance of skeletal muscle capillaries is visualized. However, one single acute bout of exercise by itself is a very strong pro-angiogenic stimulus, modifying the expression levels of various angio-adaptive factors and stimulating endothelial cell activation in skeletal muscle. When repeated chronically, such as during exercise training, these pro-angiogenic signals will ultimately lead to the formation of mature capillaries. These singular acute bouts of exercise may be considered the ‘dynamic phase’ of the angiogenic process. Over time, the expression levels of these angio-adaptive factors will be modified as the adaptation to exercise is established. Refer to figure 1.2 for a schematic depicting the possible responses of pro- and anti-angiogenic molecules in response to a bout of exercise that would favor capillary growth, and to figure 1.3 for a schematic depicting the temporal response of pro-angiogenic factors in response to repeated acute bouts of exercise leading to microvascular

adaption. The following section entitled “*molecular regulation of skeletal muscle angiogenesis*” will provide details regarding the balance between key pro- and anti-angiogenic factors.



**Figure 1.2** A single bout of exercise is a powerful stimulus to alter the angio-adaptive balance between anti- and pro-angiogenic molecules. In response to a bout of exercise, the angiogenic balance can be shifted favoring capillary growth by either lowering anti-angiogenic factors (1), elevating pro-angiogenic molecules (2), a combination of both (3), or an increase in both with a larger elevation in the pro-angiogenic factors.



**Figure 1.3** Singular acute bouts of exercise are very strong pro-angiogenic stimuli, modifying the expression levels of various angio-adaptive factors and stimulating endothelial cell activation in skeletal muscle. These singular acute bouts of exercise may be considered the ‘dynamic phase’ of the angiogenic process. Over time, as exercise bouts are repeated chronically (exercise training) the capillary network can expand by the process of exercise-induced angiogenesis.

### 1.5 Skeletal muscle capillary regression

Several conditions exist which lead to skeletal muscle capillary loss, including, but not limited to, muscle disuse, obesity, diabetes, chronic increases in blood pressure, chronic heart failure or chronic obstructive pulmonary disease (COPD) [64-66]. Researchers have employed numerous methodologies to induce skeletal muscle capillary regression in animal models including immobilization casting, nerve crushing, hindlimb or whole body suspension and motor nerve conduction blockage [65,66]. Capillary regression as a product of disuse in humans has also been studied in patients with bedrest, inflammatory myopathies, neurogenic disorders, mitochondrial myopathies, space flight and peripheral vascular diseases [66]. One theory that attempts to explain



capillary regression in the skeletal muscle tissue as a result of disuse is the over-exposure of muscle cells to oxygen, termed hyperoxia. Muscle fibers that are in a state of relative disuse become over-perfused with oxygen, resulting in capillary regression to match the metabolic demands of the muscle tissue with supply from the microvasculature. Persistent hyperoxia can lead to vasoconstriction of arterioles leading to decreased blood flow through capillaries and therefore oxygen delivery to the muscle tissue. However, vasoconstriction itself may not be sufficient to restore proper oxygenation to the muscle, and capillary regression is required [65]. Overall, it is poorly understood the process through which skeletal muscle capillaries regress in response to various disease states. Due to the unique nature of each respective pathology, the mechanisms governing molecular changes leading capillary rarefaction are vast, and require intensive research for each specific disease.

Capillary regression as a result of muscle disuse has been widely shown in the literature. Malek and colleagues [67] demonstrate the plasticity of the skeletal muscle microvasculature in response to exercise training with subsequent detraining. Rats were subjected to 10 weeks of treadmill exercise training, which as expected, resulted in increased muscle capillarity in the soleus and plantaris muscles. However, animals that underwent the exercise training but were then confined to their cages for just 7 days following the exercise period (i.e. detraining) had significantly lower capillary-to-fiber ratio and capillary density compared to the trained group. These findings highlight the rapidity that capillary regression can occur, as well as demonstrating that adaptations to exercise stimulus are not permanent and the capillary network adapts rapidly to the demands of muscle cells. Similar findings have been recorded by Huttemann et al [68] as the C/F ratio was increased following 5 weeks of treadmill training in mice plantaris muscle but 14 days detraining reversed this effect. Olenich and colleagues [69] demonstrated that mice allowed

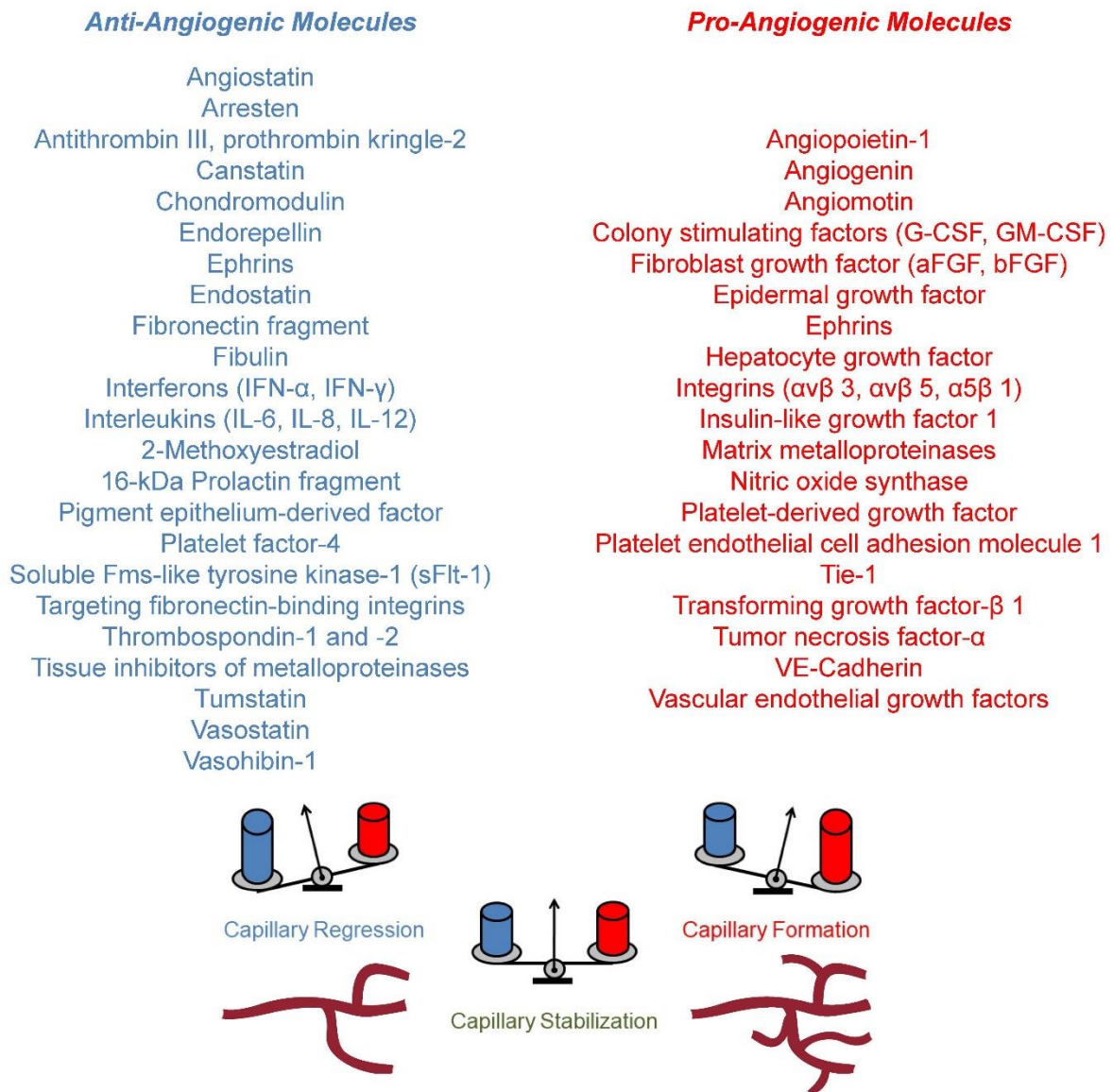
to exercise voluntarily for 3 weeks underwent exercise-induced angiogenesis with significant elevations in C/F ratio in the gastrocnemius, plantaris, and soleus muscles, however after just 7 days of detraining muscle capillarity was similar to pre-training levels. Similar findings have been described in human subjects in response to detraining, as 8 weeks of bicycle ergometer training led to a 20% increase in C/F ratio and capillary density in the vastus lateralis muscle of subjects, however 8 weeks of detraining reversed the C/F ratio similar to pre-exercise levels [70]. Additionally, a 6.3% reduction in capillary density was observed after 15 days without training in endurance runners [71]. Muscle disuse has also been demonstrated in rodent models of tail suspension which results in hindlimb unloading (mimicking space flight and muscle disuse due to the removal of natural physical forces and gravity experienced by the suspended muscles) [72]. Previous results from our laboratory have shown significant capillary regression in the soleus muscle following 9 days of hindlimb unloading [73]. Similarly, Wagatsuma et al [74] showed that 10 days of hindlimb unloading led to a 19.5% reduction in the amount of capillaries around a muscle fiber in the gastrocnemius muscle.

## **2. Molecular regulation of skeletal muscle angiogenesis**

### **2.1 *The angio-adaptive balance***

In response to metabolic and mechanical stresses, the growth of the capillary network is dependent on the expression level of various molecules that operate to either suppress or stimulate capillary growth. The angio-adaptive balance can be defined as the relative abundance, activity, and interplay between pro- and anti-angiogenic molecules. The growth, or alternatively, the regression of the skeletal muscle capillary network involves a shift in the angio-adaptive balance that is determined by alterations in expression of key stimulators and inhibitors of angiogenesis. Figure 2.1 (adapted from [64]) depicts a non-exhaustive list of identified pro- (stimulators) and

anti- (inhibitors) angiogenic molecules, respectively. Regular elevations in the expression of pro-angiogenic factors, reductions in the expression of anti-angiogenic factors, or a combination of both, can lead to expansion of the capillary network in response to training (refer to figure 1.2). As we can appreciate, there exists an extensive catalogue of angio-adaptive regulators. How do we determine which factors are the most important for skeletal muscle capillary regulation? By modulating the expression of such proteins in animal models, researchers have identified key pro- and anti-angiogenic factors. VEGF-A and Thrombospondin-1 (TSP-1) are the most widely studied pro- and anti-angiogenic factors, respectively. It has been extensively demonstrated that VEGF-A and TSP-1 are essential regulators of skeletal muscle angiogenesis, in addition to capillary regulation in a variety of tissues and organs. Our laboratory has established that the VEGF-A/TSP-1 ratio is a good indicator of the angiogenic microenvironment of the skeletal muscle [64,73,75,76]. The VEGF-A/TSP-1 ratio is defined as the comparative and relative expression level between VEGF-A and TSP-1. Since VEGF-A and TSP-1 represent essential regulatory molecules on either side of the angio-adaptive balance, the VEGF-A/TSP-1 ratio provides a key insight into the overall environment favoring either capillary maintenance, growth, or regression. The following section will provide a background review of the literature describing the regulatory role of VEGF-A and TSP-1 in skeletal muscle capillarity.



**Figure 2.1:** Diagram depicting the balance between stimulators and inhibitors of angiogenesis in the skeletal muscle. The figure lists key pro- and anti-angiogenic molecules. *Adapted from [64]*

## 2.2 Vascular endothelial growth factor-A

VEGF-A is a potent pro-angiogenic factor that is, amongst other functions, responsible for the growth of capillaries within the skeletal muscle. VEGF-A has been shown to be important for

both splitting and sprouting angiogenesis in the skeletal muscle [77]. VEGF-A is expressed by a number of different cell types including endothelial cells, myofibers, fibroblasts, macrophages, and satellite cells [7]. Several splice variants of VEGF-A exist, however in the skeletal muscle, the VEGF-A<sub>165</sub> isoform is the most abundant physiologically active [36]. The pro-angiogenic effect of VEGF-A occurs by binding to tyrosine kinase receptors including VEGF-A receptor 1 and 2 (VEGFR1 and 2) along with neuropilin-1 which serves as a co-receptor for VEGFR2, and it is VEGFR2 that is the most important for skeletal muscle angiogenesis [36]. Binding of VEGF-A to its receptors leads to a cascade of intracellular signaling pathways including the activation of extracellular signal regulated kinase 1/2 (ERK1/2) and Akt in endothelial cells which regulate a variety of cellular processes important for angiogenesis such as endothelial cell survival, proliferation, and gene expression [78,79].

VEGF-A mRNA and protein expression can be increased in response to an acute bout of exercise within human skeletal muscle [80] and interstitial VEGF-A protein levels are significantly elevated during and following exercise [81]. VEGF-A mRNA expression appears to be transiently elevated between 1 and 6 hours during post-exercise rest, returning to pre-exercise levels within 24 hours [36,82,83]. Indeed, VEGF-A mRNA expression was found to be elevated 2-4 fold at the end of a single exercise bout in rat muscle and remained elevated 4 hours afterwards, returning to baseline within 8 hours post-exercise [82]. Gavin et al [84] showed that VEGF mRNA was significantly elevated in human vastus lateralis both 2 and 4 hours following an acute bout of cycle ergometer exercise. Similarly, VEGF-A mRNA was increased in human muscle tissue by nearly two-fold following an acute bout of knee-extension exercise [85]. In accordance with increased mRNA expression, some studies have shown that VEGF-A protein levels are also elevated following a bout of exercise in human muscle [86], while others have shown no change at 1 or 2

hours post-exercise [87] or even a decrease immediately after an exercise bout [84] in human skeletal muscle. The differential response of VEGF-A protein and mRNA in response to acute exercise is further discussed in the following paragraph. While the results describing the response of VEGF-A protein to an acute bout of exercise appear somewhat controversial, studies show that basal VEGF-A protein levels are significantly higher early in an exercise training program at 10 days in human muscle [86], and at 7 days in mouse muscle [88]. Basal elevations in VEGF-A protein also appear to be transient, as no difference in basal VEGF-A protein was observed after 5 weeks of knee extension exercise compared to pre-exercise levels, despite increased basal VEGF-A protein at 10 days [89]. Similarly, Hoier and colleagues [36] have observed no changes in resting VEGF-A protein following either moderate or high intensity cycling training for four weeks in humans. In addition to alterations in VEGF-A expression, exercise also triggers elevations in its receptors. Studies have shown increased VEGFR1 and VEGFR2 mRNA 2-6 hours post-exercise in human muscle tissue [84,90]. This dual up-regulation of VEGF-A and its receptors in response to exercise serves as a mechanism for increased VEGF-A sensitivity in the skeletal muscle.

The secretion of VEGF-A by skeletal muscle cells is a mechanism through which the skeletal muscle can regulate its' own vascular supply in response to repeated bouts of exercise, thereby improving oxygen and nutrient delivery to active muscle cells. As reviewed in the previous paragraph, while some studies observe increased VEGF-A protein [86], others have shown that VEGF-A protein levels do not necessarily increase in human skeletal muscle in response to an acute bout of exercise [84]. However, it is well documented that VEGF-A concentration in muscle interstitial space increases [81] suggesting that the localization and mobilization of VEGF-A is an important response to exercise stimulus. It has been described in humans that VEGF-A is stored in vesicles distributed throughout muscle cells [36], which relocate and accumulate in the

subsarcolemmal region of following exercise [87]. This shift in subcellular location of VEGF-A vesicles coinciding with a 5 fold increase in interstitial VEGF-A protein following exercise suggests that VEGF-A is stored within vesicles in muscle cells and it secreted into the extracellular fluid in response to exercise stimulus [87]. Interestingly, VEGF-A mRNA has been shown only be elevated during rest following an exercise bout [36,82-84] yet interstitial VEGF-A protein is significantly higher early after the onset of exercise [87,91] suggesting that vesicles are pre-stored with VEGF-A, and that the elevation in VEGF-A mRNA following exercise serves to replenish VEGF-A stores within the muscle cell.

An interesting study from Birot et al [92] shows that the expression of VEGF-A could be fiber type-specific. In response to a 90 minutes exercise bout, VEGF-A protein levels were significantly higher in rat plantaris muscle compared to non-exercised muscle. Individual fibers were isolated from whole plantaris muscle, and were identified as either type I, IIa, IIx, or IIb according to the expression of myosin heavy chain isoforms. While pooled myofibers from exercised animals presented a marked increase in VEGF-A mRNA compared to controls, only type IIb fibers had elevated VEGF-A expression when observed individually. Furthermore, not all fibers expressed VEGF-A, with type IIb fibers presenting the highest proportion of fibers with VEGF-A mRNA detected. Taken together, this data suggests that VEGF-A is more abundantly produced by fast-twitch glycolytic fibers that present the largest diameter and have the lowest oxidative potential. Due to the metabolic capacity of these fibers, they may experience more exercise-induced local hypoxia compared with increasingly more oxidative fibers (i.e. type IIx, IIa, or I), thus leading to significantly more VEGF-A expression in response to an acute bout of exercise.

Although VEGF-A is produced and released by various cell types, the importance of myofiber-derived VEGF-A is evident in studies where myocyte VEGF-A production was genetically deleted. In order to delete VEGF-A in the muscle tissue, Tang and colleagues [93] utilized intramuscular injection of cre-recombinase in VEGF-AloxP(+/+) mice. VEGF-A deletion resulted in significantly reduced capillary-to-fiber ratio (-67%) and capillary density (-69%) in the gastrocnemius four weeks post-injection. Olfert et al [94] demonstrate that muscle-specific VEGF-A deletion in mice led to a 48% decrease in the capillary-to-fiber ration and a significant reduction of 39% capillary density in the gastrocnemius muscle. In another eloquent study, Olfert and colleagues [95] showed that while wild-type mice increased capillary density by 59% in the gastrocnemius muscle, and improved both maximal running speed and time to exhaustion following a 6 week training period, myocyte-specific VEGF-A deletion fully abrogated exercise-induced angiogenesis and exercise performance. Furthermore, muscle overload-induced angiogenesis was entirely lost in mice with myocyte deletion of VEGF-A [96]. Both the ligand and the receptor are important for VEGF-A induced angiogenesis, as Lloyd and colleagues [97] have shown in a rodent model that VEGFR2 blockage blunted skeletal muscle angiogenesis in response to training compared to control animals.

The production of VEGF-A by myocytes is often a result of hypoxic and metabolic stress, which are strongly associated with exercise [36,92,98]. Indeed, hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is an important transcriptional regulator of VEGF-A. HIF-1 $\alpha$  acutely senses low cellular oxygen levels and can bind to the hypoxia responsive element on the VEGF-A gene and upregulate the expression of VEGF-A in the skeletal muscle [99]. Additionally, both HIF-1 $\alpha$  and hypoxia itself also play important roles in VEGF-A regulation at the post-transcriptional level by stabilizing VEGF-A mRNA and promoting mRNA translation [99]. Interestingly, it has been shown that



Murine Double Minute-2 (Mdm2) shuttles from the nucleus to the cytoplasm during hypoxia and can bind to VEGF-A mRNA increasing its stability and translation in cancer cells [100]. The Mdm2 molecule is the main focus of this dissertation, and will be discussed at length in the following section. VEGF-A expression is also regulated by the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) [101], and it has been shown that PGC-1 $\alpha$  knockout mice had up to an 80% reduction in muscle VEGF-A protein which corresponded to a 20% decrease in muscle C/F ratio versus wild-type mice [102]. Furthermore, while wild-type mice had significantly more VEGF-A protein in the quadriceps following a 5 weeks exercise period, however this exercise-induced effect on VEGF-A was lost in the PGC-1 $\alpha$  knockout mice [102]. PGC-1 $\alpha$  appears to regulate expression of VEGF-A through the estrogen related receptor alpha (ERR $\alpha$ ) pathway [103]. The platelet-derived growth factor (PDGF) has been shown to induce elevations in VEGF-A mRNA expression and protein secretion in cultured human vascular smooth muscle cells, and that PDGF-induced VEGF-A promoter activity was dependent on AP-1 [104]. Similarly, Wang et al [105] have shown that PDGF treatment in porcine aortic endothelial cells led to dose-dependent elevations in VEGF-A mRNA, and that PDGF stimulation led to increased VEGF-A protein in the conditioned media of cultured endothelial cells. Other studies suggest that VEGF-A expression could be regulated by both AP-1 and sirtuin-1 (SIRT-1) in response to exercise, as expression of these factors preceded the exercise-induced rise in VEGF-A mRNA in human skeletal muscle [87]. Finally, AMP-activated protein kinase (AMPK) could regulate VEGF-A expression and protein release from muscle cells as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), a potent stimulator of AMPK activity, significantly elevated both VEGF-A mRNA and protein secretion in cultured C2C12 myocytes, and that transfection of a dominant-negative AMPK entirely reversed this effect [106].

### *2.3 Thrombospondin-1*

Thrombospondin-1 is a well described endogenous inhibitor of angiogenesis in the skeletal muscle. A number of anti-angiogenic roles for TSP-1 have been identified in relation to endothelial cells including the inhibition of VEGF-A activity and suppression of its bioavailability, as well as inducing endothelial cell apoptosis and inhibiting migration [107]. It has been shown that TSP-1 can prevent release of VEGF-A from the extracellular matrix, preventing the mobilization of VEGF-A and decreasing the amount of VEGF-A bound to its receptors [108]. Furthermore, TSP-1 has been shown to bind directly to VEGF-A, a mechanism which can promote VEGF-A clearing from the extracellular space, again leading to decreased VEGF-A interaction with its receptors [109,110]. Not only can TSP-1 prevent VEGF-A interaction with its receptors, TSP-1 can also inhibit the phosphorylation (i.e. activation) of the VEGF-A receptor 2 (VEGFR2), therefore inhibiting VEGF-A signaling [111]. By binding to its high-affinity receptor CD47, which is constitutively associated with VEGFR2, TSP-1 can inhibit VEGFR2 phosphorylation and downstream signalling [112]. Through the activation of several intracellular kinases, TSP-1 has been shown to lead to endothelial cell apoptosis in cultured endothelial cells [113]. It has been reported that TSP-1 can inhibit cell cycle progression through binding to very-low density lipoprotein receptor (VLDLR) in the membrane of capillary endothelial cells [114]. TSP-1 has also been demonstrated to inhibit endothelial cell migration by binding to CD36, which serves as one of several TSP-1 receptors [115,116]. Indeed, TSP-1 is demonstrably a potent negative regulator of the skeletal muscle microvasculature, as global deletion of TSP-1 in mice resulted in a significantly higher skeletal muscle capillarity that corresponded to a 67% increase in exercise endurance capacity when compared to controls [117]. An elegant study from Audet and colleagues [118] shows that chronic (14 days) delivery of ABT-510, a mimetic of TSP-1 targeting the CD36

receptor, resulted in significant reductions in the C/F ratio in the gastrocnemius, soleus, and plantaris muscles of mice, while also suppressing VEGF-A protein expression in the gastrocnemius and soleus. Interestingly, it appears that capillary regression as seen following an exercise training period with subsequent detraining is not a result of reduced VEGF-A expression, but rather an increase in basal TSP-1 expression [72] highlighting the importance of TSP-1 and the anti-angiogenic side of the angio-adaptive balance to influence capillary loss. Studies that have investigated skeletal muscle capillary loss in detrained muscle following an exercise period show that capillary rarefaction corresponded with increased TSP-1 protein levels [68,69], despite elevated VEGF-A levels. Furthermore, our laboratory has shown in a model of hindlimb unloading that capillary regression occurred in the soleus muscle in combination with a decreased VEGF-A/TSP-1 ratio due to elevations in TSP-1 but no alterations in VEGF-A [73]. However, capillary regression did not occur in the plantaris muscle where TSP-1 expression was unaltered in combination with elevated VEGF-A.

Interestingly, TSP-1 expression is elevated following an acute bout of exercise, with this response being ablated following 3 days of training [119]. These findings suggest that the temporal response of key anti-angiogenic molecules to exercise allows for fine control of the angiogenic process by potentially initiating angiogenesis along with elevations in pro-angiogenic molecules. It can be hypothesized that the angiogenic “start” signal is given by a decrease in TSP-1 in response to short-term training, concomitant with increased VEGF-A. Similarly, our laboratory has shown that forkhead box protein O1 (FoxO1), a transcription factor for TSP-1 [120], is significantly elevated immediately after (mRNA) and during rest (mRNA and protein) following an acute bout of exercise in mice [121]. However, when animals were trained over the course of 14 days, FoxO1 mRNA and protein expression was significantly attenuated, with TSP-1 protein levels following

the same pattern [121]. Interestingly, the anti-angiogenic signal of increased TSP-1 expression could possibly also serve as a natural angio-regulatory “brake” or “stop” signal following exercise-induced angiogenesis. Following an exercise training period during which the skeletal muscle capillary network has sufficiently expanded to meet the demands of active muscle cells, no further capillary growth is necessary. Indeed, Olfert et al [119] described the reinstatement of TSP-1 responsiveness to an acute bout of exercise following an 8 week training period, as TSP-1 mRNA increased 3 to 4 fold following exercise in the trained muscle.

Observations regarding TSP-1 transcriptional control are largely varied and are found to be cell and tissue type-specific, highlighting the various localized functions TSP-1 can serve in different tissues and contexts [122]. Both the complex assembled on the promoter region of TSP-1 and the promoter regions involved in the expression of TSP-1 can vary from one cell type to another [122]. TSP-1 transcriptional regulation has been most widely studied in the context of hyperglycemia. Wang et al [123] show that in response to high glucose, the upstream stimulatory factors (USFs) bind directly to the TSP-1 promoter in mesangial cells as observed by chromatin immunoprecipitation, while Dabir and colleagues [124] identified that the aryl hydrocarbon receptor (AhR) binds to the TSP-1 promoter region in human aortic endothelial cells in response to high glucose exposure. Additionally, a potentially significant role for TSP-1 in metabolic regulation has been demonstrated, with TSP-1 transcription found to be regulated by leptin in human aortic smooth muscle cells [125]. While no direct transcriptional mechanism was investigated, Negoescu et al [126] observed increased TSP-1 protein expression in cultured bovine adrenocortical cells by TGF $\beta$  treatment. In the context of this dissertation, TSP-1 expression has been suggested to be under the control of the transcription factor p53 in fibroblasts [127], and p53 expression was significantly associated with TSP-1 levels in human tumors [128]. In addition,

Roudier et al [120] show that in primary skeletal muscle microvascular endothelial cells, FoxO1 is a potent stimulator of TSP-1 expression. The authors show that deletion of FoxO1 in endothelial cells led to significant reductions in TSP-1 protein, while overexpression of FoxO1 drastically increased TSP-1 protein. Furthermore, chromatin immunoprecipitation showed that endogenous FoxO1 directly binds to promoter regions on TSP-1.

### **3. Murine Double Minute-2**

It can be appreciated that there exists a plethora of angio-adaptive molecules that regulate skeletal muscle capillary maintenance, growth, and regression (refer to figure 1.2). Amongst these, VEGF-A and TSP-1 have been clearly identified as essential pro- and anti-angiogenic molecules, respectively, that together influence the overall angiogenic microenvironment of the skeletal muscle. As researchers, we strive to identify novel signaling pathways and molecules that could deepen our understanding of skeletal muscle capillary control in response to physiological and pathological stimuli. The identification of a molecule that could regulate both sides of the angio-adaptive balance (VEGF-A and TSP-1), and is indispensable for capillary growth and maintenance is undeniably intriguing. Our laboratory has identified Murine Double Minute-2 (Mdm2) as an essential regulator of skeletal muscle angio-adaptation. The following sections will provide background information on Mdm2 including a pioneering study from our laboratory which formed the foundation of my dissertation research.

#### **3.1 *Murine Double Minute-2: novel regulator of the skeletal muscle capillary network***

In 2012, our laboratory published novel findings that describe Mdm2 as a key player in skeletal muscle angio-regulation. Transgenic Mdm2<sup>Puro/Δ7-9</sup> mice express 60% less Mdm2 versus wild-type animals, and present a significant 20% reduction in skeletal muscle capillaries at rest

[75]. In addition, while wild-type animals underwent exercise-induced angiogenesis in response to a training program, such an adaptive response was fully abolished in the Mdm2 deficient mice. In line with this finding, it was shown that total Mdm2 protein levels were found to be significantly elevated in healthy rodent muscle in response to prolonged exercise training. Furthermore, Mdm2<sup>Puro/ $\Delta$ 7-9</sup> mice displayed the loss of a physiological response to acute exercise, as an acute bout of treadmill running induced expression of the pro-angiogenic VEGF-A in wild-type mice but not in Mdm2<sup>Puro/ $\Delta$ 7-9</sup> mice. Mdm2 deficiency also led to significant elevations in FoxO1 protein levels, corresponding with enhanced TSP-1 protein expression. These key alterations in potent anti-angiogenic factors could explain the observed skeletal muscle capillary loss that was found to be independent of p53. These transgenic animals also had significantly less endothelial cell outgrowth (cell migration) than wild-types in a 3D muscle explant assay, indicating that Mdm2 is essential for the pro-angiogenic activity of skeletal muscle endothelial cells.

Taken together, these data identify Mdm2 as an essential regulator of rodent skeletal muscle angio-adaptation, both at rest (capillary maintenance) and in response to prolonged physical activity (exercise-induced angiogenesis). However, the molecular mechanisms through which Mdm2 exerts this pro-angiogenic effect remain largely unknown. It is the scope of my dissertation research to elucidate the angiogenic function of Mdm2.

### **3.2 Murine Double Minute-2: relationship with p53**

Existing literature has described a pro-angiogenic role of Mdm2, predominantly in a pathological context. Mdm2 is an E3 ubiquitin ligase that has implications during tumour growth [129]. Indeed, in tumors that overexpress the Mdm2 protein, angiogenesis is increased [130]. Mdm2 is often considered as an oncoprotein as it is most commonly known for its role as the main negative regulator of the tumor suppressor p53. The p53 protein is a transcription factor for many

target genes that exert anti-tumor effects by inducing cell cycle arrest and apoptosis [131]. Interestingly, p53 also promotes anti-angiogenic functions in endothelial cells by inhibiting the cell cycle and inducing apoptosis in these cells. It has been shown that p53 can exert anti-angiogenic effects by inhibiting the expression of VEGF-A in both normal and cancer cells [132-134]. Additionally, p53 has been shown to influence the expression of the anti-angiogenic TSP-1 [127,135]. Mdm2 and p53 bind directly between their respective N-terminal domains which promotes translocation of p53 from the nucleus into the cytoplasm [136,137]. As a transcription factor, this has important consequences for p53 activity as translocation to the cytoplasm blocks p53 interaction with its transcriptional machinery. In addition, Mdm2 can ubiquitinate p53 through its E3 ligase activity located in its C-terminal RING-finger domain thereby tagging p53 for proteasomal degradation [137-140]. Therefore, Mdm2 pro-angiogenic activity could be explained through its ability to negatively regulate p53-dependent anti-angiogenic activity.

Mdm2 is often overexpressed in tumors, where it can bind to p53 leading to p53 proteasomal degradation thus acting as a tumorigenic molecule [138]. Due to this pathological overexpression of Mdm2 and its subsequent effect on p53 abundance and activity, current anti-cancer strategies aim to inhibit the binding of Mdm2 to p53 in order to restore p53 function in tumors [139,141-143]. The binding pocket between Mdm2 and p53 was identified to be located on residues Phe19, Trp23 and Leu26 in the N-terminal of p53 and a hydrophobic cleft in the N-terminal of Mdm2, and a number of synthetic compounds have been shown to bind with higher affinity than p53 to this region of Mdm2, essentially competing for and blocking p53 binding with Mdm2 [137]. The first class of small-molecule inhibitors, termed nutlins were identified in 2004 by Vassilev et al [144]. Since, the nutlins and their successors have already undergone phase 1 clinical trials to treat cancers where wild-type p53 is expressed [137]. Secciero et al [145]

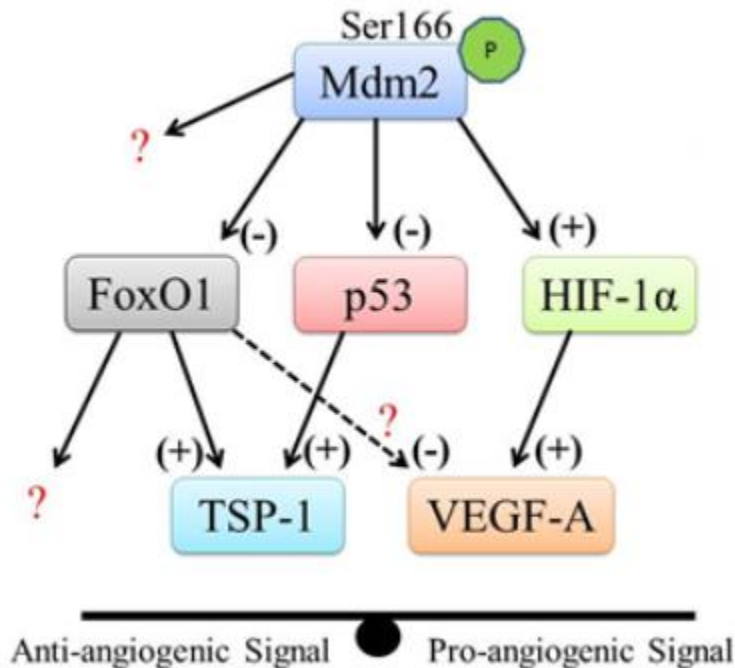
demonstrated the potent anti-angiogenic function of nutlin-3, as treatment with nutlin-3 dose-dependently prevented angiogenesis in mice with a subcutaneous matrigel plug, inhibited the formation of capillary-like structures in cultured endothelial cells, and significantly inhibited endothelial cell migration. As with other anti-cancer treatments, the delivery of nutlins and associated drugs are often administered systemically, raising concerns over potential side-effects in non-cancerous tissues, due to their highly potent anti-angiogenic effects. For this reason, one very important aim of my dissertation research was to investigate the physiological effect of Mdm2 (in healthy cells and tissues) to highlight the complexity of Mdm2 function outside of the context of cancer therapy.

### **3.3 *Murine Double Minute-2: p53-independent effects***

In contrast to its p53-dependent effects, Mdm2 may also possess other mechanisms to exert its pro-angiogenic function. Interestingly, previous studies have described that the pro-angiogenic effect of Mdm2 may however be independent of p53. Mice overexpressing Mdm2 display a higher occurrence of angiosarcoma that is characterized by excessive endothelial cell proliferation, independent of p53 [146]. Furthermore, our laboratory has shown decreased skeletal muscle capillarization in mice deficient for Mdm2, independent of the p53 background [75]. Mdm2 could indeed interact with other transcription factors targeting various angio-adaptive genes. Among these angio-adaptive molecules is the pro-angiogenic VEGF-A. It has been shown that Mdm2 can promote VEGF-A expression through interaction HIF-1 $\alpha$ , a transcription factor for VEGF-A [147-149]. In addition, we and others have show by co-immunoprecipitation that Mdm2 can bind to and negatively regulate the transcription factor FoxO1 [150,151]. FoxO1 is a transcription factor that has been shown to promote an anti-angiogenic environment by stimulating the expression of its transcriptional target TSP-1 [120]. In mice that have had endothelial cell specific deletion of



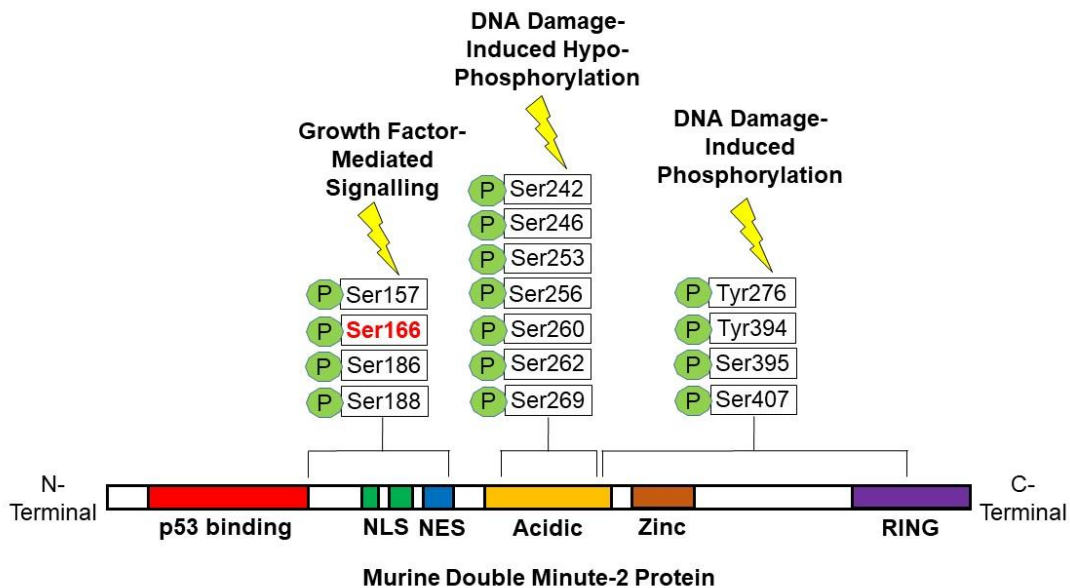
FoxO1, the onset of exercise-induced angiogenesis occurred earlier than controls [121]. Interestingly, it has also been demonstrated that overexpression of FoxO1 led to a decrease in VEGF-A mRNA levels, suggesting FoxO1 could regulate not only TSP-1, but rather both sides of angio-adaptive balance (i.e. VEGF-A and TSP-1) [152]. Thus, the role of Mdm2 in the angiogenic process appears to be very complex, suggesting that Mdm2 may represent a novel ‘master regulator’ of several transcription factors influencing the angio-adaptive balance (FoxO1, HIF-1 $\alpha$ , p53). These interactions affect both pro- and anti-angiogenic factors (VEGF-A, TSP-1), and the interplay between Mdm2 targets further highlights the complexity of the Mdm2-dependent angiogenic process. Post-translational modifications of the Mdm2 protein, such as phosphorylation (i.e. on its serine 166 [Ser166]) can enhance interactions between Mdm2 and its downstream targets, a modification of Mdm2 that will be reviewed in the following section. Refer to figure 3.1 for a schematic depicting the dynamic interactions between Mdm2 and its downstream targets.



**Figure 3.1** Mdm2 can affect both sides of the angio-adaptive balance by interactions with its downstream targets FoxO1, p53, and HIF-1 $\alpha$ , leading to altered expression of the anti-angiogenic thrombospondin-1 (TSP-1) and the pro-angiogenic vascular endothelial growth factor-A (VEGF-A). (+) indicates stimulatory and (-) indicates inhibitory effects. The letter “P” in a green circle represents a phosphorylated protein. Ser166, serine166 phosphorylation of Mdm2

### 3.4 Murine Double Minute-2: multisite phosphorylation

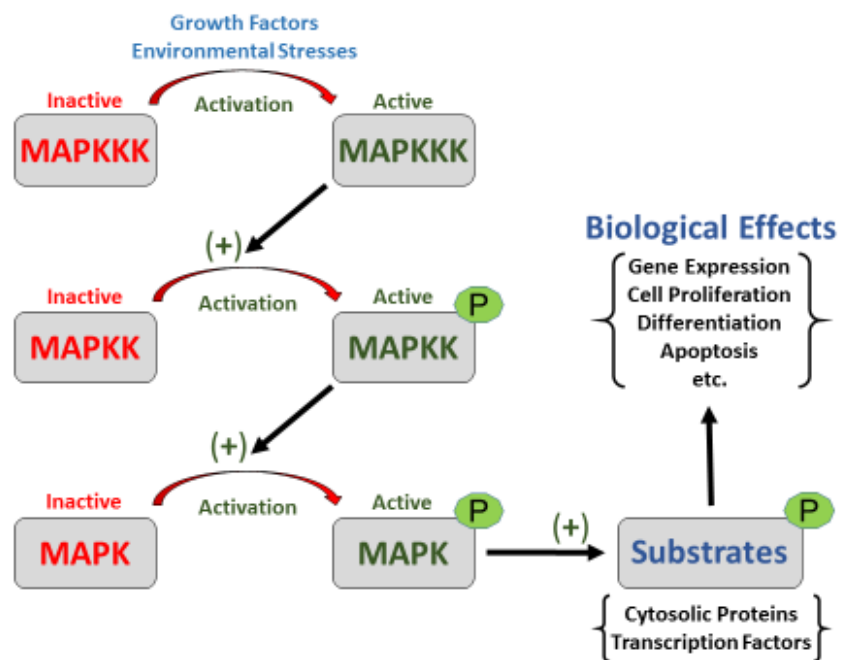
The Mdm2 protein can be subjected to multi-site phosphorylation which affects Mdm2 stabilization, activation, and localization (Figure 3.2) [153]. Phosphorylation of Mdm2 on its serine 166 (p-Ser166-Mdm2) is a signal for nuclear translocation [154], as this phosphorylation site lies in the Mdm2 nuclear localization sequence (NLS). In addition, it has been shown that p-Ser166-Mdm2 overexpression significantly increases VEGF-A expression to a greater extent than Mdm2 overexpression alone [155]. Another study demonstrated that p-Ser166-Mdm2 contributes to the ability of Mdm2 to influence HIF-1 $\alpha$  protein levels [156], which would result in increased transcriptional activity towards VEGF-A. p-Ser166-Mdm2 has also been shown to be important for Mdm2 interaction with p53 [157] since phosphorylation at this site promotes Mdm2 nuclear translocation where it can promote p53 nuclear export, ubiquitination, and proteasomal degradation [140,154]. In addition, our group has contributed to show that skeletal muscles of rats subjected to hind-limb ischemia by iliac artery ligation led to reduced p-Ser166-Mdm2 and a decreased interaction between Mdm2 and FoxO1 proteins [151].



**Figure 3.2** Mdm2 undergoes post-translational modifications by multisite phosphorylation, stimulated by growth factor mediated signaling or DNA damage causing phosphorylation or hypo-phosphorylation. Functional domains on Mdm2 are depicted and include p53 binding domain, nuclear localization sequence (NLS), nuclear export sequence (NES), acidic domain and the zinc and RING finger domains. Specific phosphorylation sites are listed. In response to growth factor mediated signaling, Mdm2 can be phosphorylated on its serine166 (shown in red) which lies in the nuclear localization sequence, thus favoring Mdm2 shuttling from the cytoplasm into the nucleus where it can interact with its key downstream targets p53, FoxO1 and HIF-1 $\alpha$  (*adapted from*[153]).

Interestingly, existing literature suggests there may be a link between VEGF-A signaling and Mdm2 phosphorylation on its serine 166. VEGF-A acts on VEGFR2 present on the surface of endothelial cells, subsequently activating downstream kinase signaling pathways including mitogen-activated protein kinases (MAPKs). MAPKs are expressed in all eukaryotic cells and include the following kinases: extracellular signal regulated kinase 1/2 (ERK1/2), extracellular signal-regulated kinase 5 (ERK5), c-Jun NH<sub>2</sub>-terminal Kinase (JNK), and p38 [158]. Figure 3.3 depicts the activation pathway responsible for the onset of MAPK signaling. The initial step is the activation of a MAP kinase kinase kinase (MAPKKK), which are regulated by membrane

recruitment, oligomerization, or phosphorylation leading to the activation of a MAP kinase kinase (MAPKK) and sequentially a MAP kinase [158]. These signaling cascades can respond to a variety of factors including environmental stressors and growth factors and can control various cellular processes including gene expression, cell proliferation, differentiation, inflammation, and apoptosis (Figure 3.3).



**Figure 3.3** Mitogen activated protein kinase (MAPK) activation cascade. In response to stimuli such as environmental stresses and growth factors, a MAP kinase kinase kinase (MAPKKK) becomes active and leads to the activation of a MAP kinase kinase (MAPKK), ultimately inducing the phosphorylation of MAP kinase (MAPK). This MAPK activation stimulates the phosphorylation of downstream substrates such as cytosolic proteins and transcription factors that have various biological effects in gene expression, cell proliferation, differentiation, and apoptosis. (+) indicates stimulatory and (-) indicates inhibitory effects. The letter “P” in a green circle represents phosphorylation of a protein.

The activation of ERK1/2 is highly dependent on Raf, a MAPKKK for ERK1/2 [159]. Raf can phosphorylate and activate MEK1 and MEK2, which are MAPKKs that stimulate the phosphorylation of ERK1/2. This occurs predominantly in response to growth factors such as insulin [160] epidermal growth factor [161], nerve growth factors [162] or other factors involved in cell growth and differentiation. ERK1/2 can then assert effects on downstream kinases either in the cytoplasm, such as the p90 ribosomal S6 kinase (p90RSK), or translocate to the cell nucleus to phosphorylate transcription factors, such as c-Myc, c-Jun, c-Fos or ELK-1 [163]. ERK1/2 are known as downstream targets of the VEGF-A/VEGFR2 signaling pathway [164]. While Akt has been demonstrated to play a main role in phosphorylating Mdm2 [154,165], it has been suggested that ERK1/2 may be involved as well [166,167]. Since there is no evidence for a direct interaction of ERK1/2 and Mdm2, p90RSK may be the link for this ERK1/2 effect on Mdm2. p90RSK can be phosphorylated by ERK1/2 [168], and has been shown to phosphorylate Mdm2 *in vitro* [169]. Interestingly, a paper from Seko and colleagues [170] has shown that VEGF-A stimulation induced the activation of MAP kinases and p90RSK in cultured rat cardiac myocytes and fibroblasts. Furthermore, it has been shown that ERK1/2 can be phosphorylated by acute bouts of exercise which in combination with elevated VEGF-A protein (see previous section on VEGF-A in exercise-induced angiogenesis) further suggests a potential relationship between VEGF-A and ERK1/2 activation. Both mouse and rat models of exercise show an increase in ERK1/2 phosphorylation in the skeletal muscle [165,171,172]. These results are paralleled in the skeletal muscle of both trained and untrained human subjects in response to cycling exercise [173-175], and both endurance and resistance exercise were found to be sufficient to induce ERK1/2 activation [164,176,177]. Furthermore, isolated rat and mouse skeletal muscles that were

stimulated to contract *in vitro* underwent significant elevations in ERK1/2 phosphorylation [168,169,178,179].

#### **4. Type 1 diabetes and skeletal muscle microvasculature**

While Mdm2 is predominantly studied in the context of cancer due to its negative regulation of the tumor suppressor p53, Mdm2 could also play a significant role in other pathologies affecting the skeletal muscle capillary network, since it is now clear that Mdm2 possesses p53-independent functions. Hyperglycemia has numerous deleterious effects on endothelial cells and can lead to microvascular complications and co-morbidities. Specifically, type 1 diabetes (T1D) is highly understudied in the context of skeletal muscle capillarization, with very few studies available investigating angio-adaptation of the skeletal muscle in response to diabetic hyperglycemia. Indeed, skeletal muscle capillaries could also be susceptible to detrimental adaptations as a result of type 1 diabetic hyperglycemia.

##### **4.1 Murine Double Minute-2 in diabetes**

Previous results from our laboratory suggest that altered Mdm2 function may play an important role in the pathological adaptation to diabetes. It was shown that in Zucker diabetic fatty (ZDF) rats, a common model of type-2 diabetes, Mdm2 protein levels are significantly lower than in lean controls [75]. In addition, FoxO1 protein levels were elevated in the ZDF rats compared to controls. This decrease in Mdm2 and elevated FoxO1 proteins occurred concomitantly with significant loss of skeletal muscle capillaries. Interestingly, voluntary exercise training in ZDF rats rescued the loss of capillaries and restored Mdm2 protein levels to that of healthy control animals. Moving from type-2 to type 1 diabetes, I have contributed to show for the first time that Mdm2 protein is significantly suppressed in the skeletal muscle of streptozotocin-induced type 1 diabetic

rats compared to non-diabetic controls, coinciding with a significant loss in skeletal muscle capillaries (see appendix for *academic research paper 5* [180]). Taken together, it is evident that Mdm2 is affected by the diabetic state and observed alterations in Mdm2 protein are associated with decreased skeletal muscle capillarization in diabetic animal models.

#### **4.2 Endothelial dysfunction in type 1 diabetes**

A serious cause of endothelial cell dysfunction is persistent and uncontrolled high blood glucose levels, or hyperglycemia, as a result of diabetes. Type 1 diabetes, also known as insulin-dependent diabetes, is a disease that occurs in response to both genetic and environmental factors [181]. T1D triggers an autoimmune response that destroys pancreatic  $\beta$ -cells within the islets of Langerhans resulting in loss of insulin production and secretion, leading to high blood glucose levels (hyperglycemia) and reliance on exogenous insulin administration [182]. Although T1D only accounts for roughly 5-10% of all diabetes cases, it can contribute to as much as 40% of healthcare costs associated with diabetes due to the early age of onset [181,182]. Therefore, understanding the alterations in physiological vascular functioning that occurs in T1D is of great importance for both the treatment and prevention of diabetes-associated vascular pathology.

Diabetes is associated with both macro- and micro-vascular complications, which represent the main causes of mortality in individuals with T1D, especially in patients living with the disease for 5 or more years [183]. Within these patients, retinopathy (eye disease), neuropathy (neural damage), and nephropathy (kidney disease) are the major micro-vascular diseases, evident in small blood vessels (i.e. capillaries). The causes of vascular complications are complex and varied, and typically include a combination of prolonged hyperglycemia leading to endothelial degeneration or (paradoxically) hyper-vascularization, alterations in vascular permeability, and changes in blood pressure [182]. Diabetic retinopathy, for instance, can be separated into two stages of

development (non-proliferative and proliferative) and is the leading cause of blindness in adults aged 20-74 years [184]. The non-proliferative phase entails the thickening of the retinal basement membrane, altering vascular permeability and therefore the blood-retinal barrier [185]. The proliferative phase is defined by the degeneration or occlusion of retinal capillaries, leading to hypoxia and the subsequent release of pro-angiogenic factors that induce neovascularization and the accumulation of fluid within the retina contributing to visual impairment [182]. Another prevalent disease in T1D is diabetic neuropathy, and is associated with microvascular dysfunction such as basement membrane thickening and endothelial hyperplasia leading to a hypoxic environment for neuronal cells [182]. However, recent research suggests that the observed vascular abnormalities are secondary to neural and glial disorders, and that diabetic neuropathy selectively targets sensory and autonomic neurons over motor neurons with minimal involvement of the vascular system [182]. Finally, diabetic nephropathy is characterized by a decrease in glomerular filtration rate and the development of proteinuria [182]. Nephropathy is associated with the onset of hypertension, and is a major risk factor for the development of macro-vascular complications such as heart attacks and strokes in T1D individuals [186].

#### **4.3 *Skeletal muscle angio-adaptation in type 1 diabetes***

To date, the majority of the literature available on the microvascular complications that arise in patients with T1D focuses on the traditional ‘big three’ of diabetes-induced diseases; retinopathy, neuropathy and nephropathy. Far less research exists describing how T1D affects the skeletal muscle fibres and the skeletal muscle capillary network. However, the existing literature indeed suggests capillary rarefaction and alterations in angio-adaptive molecules. Kivelä and colleagues [187] demonstrate that T1D induction by streptozotocin injection significantly decreased mRNA levels of VEGF-A, VEGF-A receptor-1 (Flt-1) and VEGF-A receptor-2 (Flk-1)



in samples of the mouse calf muscle complex (gastrocnemius, soleus and plantaris). In addition to the T1D-induced down-regulation of pro-angiogenic factor expression, mRNA levels of TSP-1 were significantly higher in diabetic mice compared to healthy controls. Interestingly, a 5-week exercise program partially restored the diabetes-induced reduction in VEGF-A mRNA, however was unable to reverse the increase in TSP-1 expression seen in sedentary diabetic mice. In accordance with VEGF-A mRNA expression, VEGF-A protein levels were significantly lower at both 3 and 5 weeks following the onset of diabetes in sedentary mice. Exercise training served to delay the reduction in VEGF-A protein levels, however trained diabetic mice still displayed significantly lower VEGF-A protein levels after 5 weeks of training compared to healthy controls. Interestingly, there was no difference in the capillary-to-fiber ratio between trained diabetic and untrained diabetic mice, and both groups had a significant reduction in skeletal muscle capillarization compared to non-diabetic controls. Similarly, Wallberg-Henricksson et al [188] report that while healthy male subjects increased the capillary-to-fiber ratio and capillary density in the gastrocnemius muscle in response to an 8 week endurance exercise training, these effects were lost in subjects with T1D. While it has been demonstrated that individuals with type 1 diabetes can benefit from exercise training as seen by increased oxygen uptake and improved muscle strength, muscle tissue perfusion suffered in diabetics, as capillary growth did not match exercise-induced muscle hypertrophy [189]. Other studies indeed have shown suppressed muscle perfusion in type 1 diabetics [190,191], which may be a result of an enlargement of the capillary basement membrane [190]. The skeletal muscle may not be the only striated muscle in which the capillary network is detrimentally affected by diabetes, as it has been shown that coronary collateral vessel development is impaired diabetics compared to healthy individuals [192].

Taken together, very little research exists examining the impact of type 1 diabetes on the skeletal muscle microvasculature. However, an intriguing research area emerges as our laboratory has clearly identified Mdm2 as an essential regulator of skeletal muscle angiogenesis and capillary maintenance, and have demonstrated that Mdm2 protein levels are sensitive to the diabetic state corresponding with the loss of skeletal muscle capillaries. Therefore, Mdm2 appears to be an attractive target to study in the pathological context of type 1 diabetes.

## 5. OBJECTIVES & HYPOTHESES

The global objective of my research is to elucidate the complex pro-angiogenic role of Mdm2 in both physiological (in response to exercise) and pathological (in response to disease states such as diabetes) contexts. Through the implementation of various experimental models including the investigation of Mdm2 in rodent and human skeletal muscle as well as in primary endothelial cell culture, my research aims to deepen our understanding of skeletal muscle capillary angio-adaptation. Specific research aims and hypotheses are presented below:

1) Mdm2 has historically been studied in the context of cancer due to its negative regulation of p53. However, previous research from our laboratory has suggested an important physiological role of Mdm2 in healthy tissue such as the skeletal muscle. It was demonstrated that Mdm2 is essential for both capillary maintenance and for exercise-induced angiogenesis to occur in the skeletal muscle. Furthermore, it was shown in rodents that total Mdm2 protein in muscle is elevated following a prolonged exercise training period [75]. My first research objective was to determine whether this specific finding that Mdm2 protein levels are increased in response to training could be translated to human subjects. I hypothesized that similarly to what was observed in rodents, prolonged exercise training will also increase total Hdm2 (the human analogue of Mdm2) protein expression in human skeletal muscle.

2) It has been well documented that Mdm2 can undergo post-translational modification by multisite phosphorylations. Specifically, phosphorylating Mdm2 on its serine 166 (p-Ser166-Mdm2), which lies in the Mdm2 nuclear localization sequence, promotes Mdm2 translocation from the cytoplasm into the nucleus where it can interact with its key downstream targets p53, FoxO1 and HIF-1 $\alpha$ . I hypothesize that Mdm2 will undergo phosphorylation (i.e. activation) in

response to an acute bout of exercise in rodent and human skeletal muscle tissue. Second, I aim to determine whether Mdm2 activation could lead to altered binding interactions between Mdm2 and its downstream targets in primary human endothelial cells. Finally, I hypothesized that this activation of Mdm2 by phosphorylation will have direct effects on the pro-angiogenic activity of those endothelial cells (i.e. migration).

3) Previous results from our laboratory indicate that Mdm2 protein level is associated with capillary loss in a rodent model of type-2 diabetes [75]. It has been widely reported that type 1 diabetes (T1D) leads to microvascular pathology, while only few studies have shown skeletal muscle capillary regression in rodent models. Those studies that have investigated the effect of type 1 diabetes on the skeletal muscle microvasculature in animal models have exclusively utilized the easy and cost-effective approach of chemically-inducing diabetes by systemic streptozotocin (STZ) injection. However, the onset of overt hyperglycemia in STZ-treated animals is rapid (as little as 48-72 hours following injection) with many potential systemic adverse effects from STZ itself on various cell types and tissues including skeletal muscle [193-196]. However, BioBreeding (BB) diabetic rats spontaneously develop T1D between 50-90 days of age, around the time of puberty, and present characteristics that closely resemble those of human patients with T1D [197-201]. I therefore hypothesized that, similar to the observed effects in a rodent model of type 2 diabetes, T1D will lead to downregulation of Mdm2 protein levels in BB diabetic rats. As Mdm2 signaling is highly complex with Mdm2 able to interact with several downstream targets to affect both sides of angio-adaptive balance, I hypothesized that the downregulation of Mdm2 in the diabetic state could result in altered expression levels of VEGF-A, FoxO1, and TSP-1. Any alterations in these key angio-adaptive molecules could contribute to capillary regression in the muscle of type 1 diabetic BB rats.

## 6. ACADEMIC RESEARCH PAPER 1

**Novel perspective: exercise training stimulus triggers the expression of the oncoprotein human double minute-2 in human skeletal muscle**

Emilie Roudier, **Julian Aiken**, Dara Slopack, Fares Gouzi, Jacques Mercier, Tara L. Haas, Thomas Gustafsson, Maurice Hayot & Olivier Birot

*Physiol Rep.* 2013 Jul;1(2):e00028. doi: 10.1002/phy2.28.

### Author contributions

Julian Aiken:

- Contributed to western blotting analysis
- Contributed to data analysis
- Contributed in the preparation of the manuscript and figures

### Scientific context:

- Our laboratory has demonstrated in a rodent model of Mdm2 deficiency that Mdm2 is essential for skeletal muscle capillary maintenance, exercise-induced angiogenesis, and the pro-angiogenic response to an acute bout of exercise [75]
- Mdm2 is considered to be highly oncogenic due to its interaction with p53, however the loss of capillaries in Mdm2 deficient mice was found to be independent of p53 background
- Among several other novel findings, it was shown that Mdm2 protein levels are elevated following prolonged exercise training in the skeletal muscle of wild-type rats

- These findings suggest a physiological role of Mdm2 in the skeletal muscle

**Research aims:**

- Determine whether the previous findings that Mdm2 protein is elevated following exercise in rodents could be translated to human skeletal muscle following an exercise training program

**Summary of main findings:**

- In agreeance with rodent models, Hdm2 (the human analogue of Mdm2) protein is elevated in human skeletal muscle in response to a 6-week exercise training program
- The increase in Hdm2 was found to be consistent across young and senior subjects, men and women, and those with varying fitness levels
- Despite Hdm2 being predominantly studied in the context of cancer, we show that Mdm2 is sensitive to the *physiological* stimulus of exercise training, and may play an important role in human skeletal muscle as a key regulator of angiogenesis

**Novel perspective: exercise training stimulus triggers the expression of the oncoprotein  
human double minute-2 in human skeletal muscle**

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Running head: Hdm2 Expression in Exercised Human Skeletal Muscle

## **Abstract**

High expression levels of human double minute-2 (Hdm2) are often associated with increased risk of cancer. Hdm2 is well established as an oncoprotein exerting various tumorigenic effects. Conversely, the physiological functions of Hdm2 in nontumor cells and healthy tissues remain largely unknown. We previously demonstrated that exercise training stimulates expression of murine double minute-2 (Mdm2), the murine analog of Hdm2, in rodent skeletal muscle and Mdm2 was required for exercise-induced muscle angiogenesis. Here we showed that exercise training stimulated the expression of Hdm2 protein in human skeletal muscle from +38% to +81%. This robust physiological response was observed in 60–70% of the subjects tested, in both young and senior populations. Similarly, exercise training stimulated the expression of platelet endothelial cell adhesion molecule-1, an indicator of the level of muscle capillarization. Interestingly, a concomitant decrease in the tumor suppressor forkhead box O-1 (FoxO1) transcription factor levels did not occur with training although Mdm2/Hdm2 is known to inhibit FoxO1 expression in diseased skeletal muscle. This could suggest that Hdm2 has different targets when stimulated in a physiological context and that exercise training could be considered therapeutically in the context of cancer in combination with anti-Hdm2 drug therapies in order to preserve Hdm2 physiological functions in healthy tissues.



## **Introduction**

Expression or function of the tumor suppressor p53 protein is statistically altered in about 50–60% of human cancers (Soussi and Wiman 2007). The E3 ubiquitin ligase murine double minute-2 (Mdm2) is an oncoprotein mostly known for its negative regulatory role on p53 function, inhibiting p53 transcriptional activity, promoting its nuclear export, and targeting it for proteosomal degradation (Marine and Lozano 2010; Li and Lozano 2013). Interestingly, Mdm2 is often overexpressed in human cancers and can also exert some tumorigenic activity independently of p53 (Marine and Lozano 2010). For example, Mdm2 activity contributes to enhance cell proliferation and to suppress the cell cycle arrest and apoptosis by regulating various molecules such as the retinoblastoma protein, E2F transcription factors, p21, or XIAP. Also, Mdm2 involvement in promoting tumor angiogenesis and inflammation has recently emerged through its implication in vascular endothelial growth factor-A (VEGF-A) proangiogenic and NF- $\kappa$ B proinflammatory signaling (Nieminen et al. 2005; Carroll and Ashcroft 2008; Busuttill et al. 2010; Thomasova et al. 2012). With such broad and complex tumorigenic effects, it is not surprising that a search in the Pubmed database indicates that 90% of Mdm2-related publications refer to a tumor context.

Among current anti-cancer strategies, some aim to inhibit Mdm2 function including downregulating its expression level, inhibiting its ubiquitin ligase activity, and blocking its interaction with p53 (Vassilev 2007; Li and Lozano 2013). Clinical trials are even already in place (<http://www.clinicaltrials.gov>). Of importance, the administration of anti-Mdm2 drugs in animal models and even in clinical trials is usually performed with systemic delivery (Secchiero et al. 2011), raising the unanswered question of non-negligible risks of deleterious side effects for healthy tissues.

Transgenic models have clearly established that full deletion of Mdm2 is lethal (Toledo and Wahl 2006). Mendysa et al. (2006) generated  $Mdm2^{Puro/\Delta7-9}$  transgenic mice harboring knockout and hypomorphic alleles for Mdm2. As a consequence these animals express only 40% of Mdm2 compared with the wild-type littermates.  $Mdm2^{Puro/\Delta7-9}$  mice show no embryonic lethality and are protected against tumorigenesis.

We have recently explored further the vascular phenotype of the  $Mdm2^{Puro/\Delta7-9}$  mice and demonstrated that Mdm2 plays a key physiological role in regulating rodent skeletal muscle capillarization. In sedentary  $Mdm2^{Puro/\Delta7-9}$  mice, the level of muscle capillarization was decreased by 20% (Roudier et al. 2012). Interestingly, whereas prolonged endurance training stimulated Mdm2 protein expression and promoted angiogenesis in wild-type muscles, the growth of new capillaries was blunted in  $Mdm2^{Puro/\Delta7-9}$  mice.

To our knowledge, this is one of very few characterizations of a physiological role for Mdm2 in an adult and healthy tissue. Skeletal muscles account for about 40% of our body weight, ensuring key functions from locomotion to metabolic regulation of glycemia. By matching the blood supply to the metabolic demand of active myofibers, the capillary network is a crucial determinant of muscle function (Egginton 2009). Thus, if inhibiting Mdm2 expression or function in tumor cells is an appealing anti-cancer strategy, we can, however, question what could be the consequence of systemic targeting for striated muscles, including respiratory muscles, diaphragm, skeletal muscles, and cardiac muscle, with an impact on vital functions such as locomotion, blood circulation, respiration, and metabolic homeostasis.

Exercise training has been established as an efficient, practical, and costless approach to minimize side effects of anti-cancer therapies in healthy tissues (Mishra et al. 2012). In the context of Mdm2, the use of rehabilitating exercise training could therefore be considered in combination

with anti-Mdm2 drugs to preserve its physiological (and required) level of expression in healthy tissues.

Here, we hypothesize that the stimulatory effect of exercise training on Mdm2 muscle expression observed in rodent models will translate into human double minute-2 (Hdm2) protein, the human analog of the murine Mdm2, in human skeletal muscle.

## **Methods**

*Ethical approval.* All research protocols conformed to the standards of the latest revision of the Declaration of Helsinki and were approved by local institutions, respectively, the ethics committee at the Karolinska Institutet (Stockholm, Sweden) for the training of young male subjects, and the ethics committee from Montpellier University Hospitals for the training of the senior population. Informed written consent was obtained from all subjects.

*Participants.* Two populations of subjects were studied. Sixteen sedentary young male subjects, all healthy and without any medications, were recruited. The subjects did not undertake any regular sporting activities in the 6 months prior to the 6-week training program. These subjects were part of a larger study involving a total of 24 subjects (Keller et al. 2011). Fourteen senior subjects (seven men and seven women) aged from 50 to 75 years with no disease and less than 150 min of moderate-to-vigorous physical activity per week were recruited (Gouzi et al. 2013). The clinical characteristics of all subjects including age, body weight, height, body mass index, and peak oxygen consumption ( $\text{VO}_2$ ) are summarized in Table 1.

*Exercise training protocols.* Young male subjects performed an incremental cycloergometric test until exhaustion on an electrically braked cycloergometer (RE 990, Rodby innovation, Vange,

Sweden), following the individualized protocol and according to the international standards (Ross 2003). During the exercise test, heart rate, ECG, blood pressure, and transcutaneous oxygen saturation were monitored. Oxygen consumption ( $\text{VO}_2$ ) and carbon dioxide production ( $\text{VCO}_2$ ) were measured and calculated by breath-by-breath analysis (Sensormedics, Vmax 229, Autobox, Yorba Linda, CA). Maximal power output was the maximal workload sustainable, and peak oxygen consumption (peak  $\text{VO}_2$ ) was the mean value during the last 20 sec of the test. At peak  $\text{VO}_2$ , the respiratory exchange ratio exceeded 1.10 on all occasions. The training protocol consisted of 24 sessions of 45-min cycling endurance exercise, condensed in 6 weeks, four times per week at an intensity corresponding to 70% of the pretraining peak  $\text{VO}_2$  (100% compliance). Senior subjects performed an incremental cycloergometric test until exhaustion on an electrically braked cycloergometer (Ergoselect 200P, Ergolyne, Bitz, Germany), following the individualized protocol and according to the international standards (Ross 2003). During the exercise test, heart rate, ECG, blood pressure, and transcutaneous oxygen saturation were monitored. Oxygen consumption ( $\text{VO}_2$ ) and carbon dioxide production ( $\text{VCO}_2$ ) were measured and calculated by breathby-breath analysis (Sensormedics, Vmax 229, Autobox, Yorba Linda, CA). The ventilatory threshold was blindly and independently assessed for each subject by two experienced practitioners on the basis of noninvasive methods (ventilator equivalent and V-slope methods), as recommended (Ross 2003). The training protocol consisted of 20 sessions of 45-min cycling endurance exercise, condensed in 6 weeks, three times per week at an intensity corresponding to the subject's ventilatory threshold, and corresponding to  $60 \pm 5\%$  of the pretraining peak  $\text{VO}_2$  (Nici et al. 2006). This intensity was continuously monitored with a cardiofrequency meter. Each session was completed by 30 min of strength building exercise (8–10 exercises, with sets of 10–

15 repetitions). All training sessions were supervised by an experienced clinician to ensure the compliance of subjects.

*Muscle biopsies.* Vastus lateralis muscle biopsies were performed pretraining and 24 h after the last training session as previously described (Hayot et al. 2005; Keller et al. 2011). Muscle samples were dissected free of visible connective tissue and fat and the muscle tissue was immediately frozen in isopentane cooled to freezing point with liquid nitrogen, and stored at -80°C until analysis.

*Western blotting.* Immunoblotting was carried out on protein extracts from muscle tissue as previously described (Milkiewicz et al. 2011; Roudier et al. 2012). Proteins were extracted from muscle tissue using a protein lysis buffer containing 1 mg/mL phenylmethylsulfonyl fluoride, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L NaF (Sigma, Montreal, Canada), and 19 protease inhibitors (Complete Mini and PhosStop tablets from Roche Diagnostics, Laval, Canada). Twenty to 40 mg of frozen muscle was mixed at 4°C with lysis buffer (15 volumes of RIPA per mg of muscle). For each sample, protein extracts were prepared using two stainless carbide beads (Retsch, Fisher Scientific, Montreal, Canada) in the Retsch MM400 tissue lyser (2 x 30 pulses/sec, Retsch GmbH, Haan, Germany). Denatured samples (20–30 µg/well) were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and blotted onto nitrocellulose (Whatman, BA95, Sigma-Aldrich, Oakville, Ontario, Canada) membranes. Quality of the transfer was confirmed by Ponceau S red staining. After blocking with 5% fat-free milk at room temperature for 45 min, the blots were probed overnight at 4°C with primary antibodies against the following proteins: endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1) (clone JC70A, cat. M0823, Dako, Burlington, Ontario, Canada), Mdm2/Hdm2 (clone 2A10, supernatant from the hybridoma previously described in Chen et al. [1993]), forkhead box O-1 (FoxO1)

transcription factor (clone C29H4, cat. #2880; Cell Signaling Technology, Danvers, MA), and  $\beta$ -actin (clone C4, cat. Sc-47778; Santa Cruz Biotechnologies, CA).  $\beta$ -actin was detected as a loading control. Proteins were visualized using an enhanced chemiluminescence procedure (SuperSignal West Pico, #34080; Thermo Scientific, Nepean, Ontario, Canada, or Millipore Immobilon #WBKLS0100; Thermo Scientific) and a Kodak Imaging station 4000MM Pro. Western blot images were analyzed with Carestream Molecular Imaging software. For each population of subjects, samples were randomly loaded on gels including a calibrator (i.e., loading of a protein extract sample composed of a pool of all samples) in order to conduct inter-gel comparisons.

*Statistical analysis.* Analyses were performed using Prism5 software (GraphPad). Data are represented as means  $\pm$  SEM. Two populations were considered: young men (n = 16) and senior subjects (n = 14). The effect of age and exercise training on the levels of expression of Hdm2, PECAM-1, and FoxO1 proteins as well as the peak  $\text{VO}_2$  was analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni posttests. Results were considered statistically significant when  $P \leq 0.05$ .

## **Results**

*Exercise training improves fitness level and increases muscle endothelial content.* The maximal oxygen consumption (peak  $\text{VO}_2$ ) is a good indicator of the cardiovascular fitness level. Young subjects presented higher pretraining and posttraining levels of peak  $\text{VO}_2$  than the seniors (Table 6.1, pretraining:  $49.7 \pm 1.3$  vs.  $25.7 \pm 1.6$  mL kg<sup>-1</sup> min<sup>-1</sup> [+93%], posttraining:  $56.7 \pm 1.8$  vs.  $28.6 \pm 1.6$  mL kg<sup>-1</sup> min<sup>-1</sup> [+98%],  $P < 0.001$ ). Exercise training increased the peak  $\text{VO}_2$  similarly in young and senior subjects (respectively, +14% and +11%,  $P < 0.01$ ). The level of muscle vascularization is considered to be an important determinant of exercise capacity (Wagner 2010) and we have previously shown that the level of expression of the PECAM-1 protein reflects

the level of rodent skeletal muscle vascularization (Roudier et al. 2009, 2012). Representative immunoblots for PECAM-1 pre- and posttraining expression levels in skeletal muscles from young and senior subjects are shown on Figure 6.1A and B. Following exercise training, PECAM-1 protein expression in skeletal muscle was increased in both populations, respectively, by 129% in young men and 72% in the senior subjects (Fig. 6.1C,  $1.00 \pm 0.15$  vs.  $2.30 \pm 0.34$  arbitrary units in young men [ $n = 16$ ];  $1.00 \pm 0.14$  vs.  $1.72 \pm 0.20$  arbitrary units in senior subjects [ $n = 14$ ],  $P < 0.0001$ ). Although the group of young subjects was only comprised of men, stratified analyses did not show any evidence of differences between senior men and women so results are pooled across gender for the senior group. No effect of age was observed between young and senior populations.

*Hdm2 protein expression in skeletal muscle increases with exercise training independently of age.* Representative immunoblots for Hdm2 pre- and posttraining expression levels in skeletal muscles from young and senior subjects are shown on Figure 6.2A and B. Hdm2 protein expression was significantly increased by 38% in response to exercise training in young male subjects and by 81% in the senior population (Fig. 6.2C,  $1.00 \pm 0.07$  vs.  $1.38 \pm 0.16$  arbitrary units in young men [ $n = 16$ ];  $1.00 \pm 0.11$  vs.  $1.81 \pm 0.29$  in senior subjects [ $n = 14$ ],  $P = 0.0011$ ). No effect of age or gender was detected, respectively, between young and senior populations or between senior men and senior women. Analysis of individual Hdm2 variations in response to exercise training revealed similar proportions of Hdm2 responders (i.e., Hdm2 increase posttraining) between young and senior subjects (respectively, 63% and 71%, Fig. 6.2D and E).

*Exercise training had no effect on protein expression level of Hdm2 target FoxO1.* In either young or senior subjects, exercise training had no significant effect on FoxO1 protein expression (Fig. 6.3, young men:  $1.00 \pm 0.10$  pretraining vs.  $1.06 \pm 0.16$  posttraining; senior subjects:  $1.00 \pm 0.16$  pretraining vs.  $0.98 \pm 0.16$  posttraining).

## Discussion

Our study identified exercise training as a stimulator of Hdm2 expression in human skeletal muscle. This is a robust physiological response that occurs in 60–70% of the population independently of age and gender. It also illustrates that our previous finding of a stimulatory effect of exercise training on Mdm2 levels in rodent muscle translate to humans.

Concomitantly with the increase in Hdm2, exercise training also stimulated the expression of PECAM-1, a good indicator of the level of endothelial material (Roudier et al. 2009, 2012). This finding is in line with our previous study identifying Mdm2 as an important regulator of skeletal muscle capillarization in Mdm2<sup>Puro/Δ7-9</sup> mice and showing that the reduction in Mdm2 expression restrained the proliferative and migratory response of skeletal muscle endothelial cells to the exercise stimulus (Roudier et al. 2012).

Demonstrating that exercise stimulus increases Hdm2 protein levels in human skeletal muscle might have important clinical consequences. Hdm2 is considered as an oncoprotein based on its various tumorigenic functions, and particularly its role as the main negative regulator of the tumor suppressor p53 (Li and Lozano 2013). The development of Mdm2/Hdm2 inhibitors is a very attractive approach to restore p53 function in cancer cells and to inhibit Mdm2-mediated tumor angiogenesis (Vassilev 2007; Millard et al. 2011 Li and Lozano 2013). In fact, clinical trials are already under development (Vassilev 2007; Millard et al. 2011).

The ability to stimulate Hdm2 muscle expression using rehabilitating exercise training might thus represent an easy and practical approach to preserve Hdm2 function in skeletal muscles from cancer patients undergoing anti-Hdm2 therapy. In particular, Hdm2 response to exercise training was as strong in the senior population, equally composed of men and women and trained



at a very moderate intensity, as in young male subjects. The ability of the exercise training stimulus to increase Hdm2 muscle expression is therefore independent of the age, the gender, and the level of activity of the subjects, which makes the concept of combining anti-Hdm2 cancer therapies with rehabilitating exercise training very realistic. In line with this idea, physical activity is known to improve the overall quality of life and to decrease fatigue in cancer patients (Mishra et al. 2012), whereas most current anti-cancer therapies can unfortunately induced damages in non-cancer cells (Ballard-Barbash et al. 2012). Therefore, triggering Hdm2 expression by exercise training in healthy tissues might have strong antiapoptotic and survival effects in non-cancer cells, protecting them from anti-cancer therapy side effects.

Aside from the context of cancer, Hdm2 could also represent a new therapeutic target to stimulate skeletal muscle angiogenesis in chronic metabolic diseases associated with capillary regression such as obesity, diabetes, and limb ischemia. This is consistent with our previous works showing some alterations of Mdm2 expression or activation in rodent models of type-2 diabetes and limb ischemia (Milkiewicz et al. 2011; Roudier et al. 2012).

A balance between pro- and antiangiogenic factors tightly controls muscle angioadaptation, that is, the process regulating capillary maintenance, regression, or growth (Olfert and Birot 2011). Interestingly, Hdm2/Mdm2 could potentially affect both sides of the angioadaptive balance. Mdm2 was suggested to enhance the expression of the proangiogenic VEGF-A (Nieminen et al. 2005; Carroll and Ashcroft 2008). We recently showed in skeletal muscles from Mdm2<sup>Puro</sup>/D7-9 mice that Mdm2 was indispensable for the increase expression of VEGF-A in response to acute exercise (Roudier et al. 2012). Mdm2 could also contribute to restrain p53-mediated antiangiogenic effects. For example, p53 stimulates the expression of thrombospondin-1 (TSP-1) (Dameron et al. 1994), a key antiangiogenic regulator of muscle

angioadaptation (Olfert and Birot 2011). Interestingly, Mdm2 could also regulate TSP-1 independently of p53. We have shown that Mdm2<sup>Puro/Δ7-9</sup> mice express higher muscle levels of a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS-1) expression (Roudier et al. 2012), a protein enhancing TSP-1 cleavage and promoting its antiangiogenic activity (Lee et al. 2006).

Several other angioadaptive molecules might be under the control of Mdm2 such as the FoxO1 transcription factor that is known to exert antiangiogenic effects (Milkiewicz et al. 2011; Roudier et al. in press) and to be negatively regulated by Mdm2 (Fu et al. 2009). FoxO1 is also considered a tumor suppressor (Arden 2007). Interestingly, in this study, the increase in Hdm2 expression in trained human skeletal muscle was not accompanied by a decrease in FoxO1. This suggests that an increase in Hdm2 in a healthy tissue in response to a physiological stress might not be associated with an increase in its oncogenic function.

In summary, our results showed that Hdm2 expression in human skeletal muscle was increased in response to exercise training concomitantly with higher levels of capillarization. This observation provides new insight into the mechanisms by which physical activity might improve muscle function. Further studies are required to investigate the underlying mechanisms by which exercise training modulates Hdm2 and its targets. A better understanding of the molecular events that regulate Hdm2 in non-pathological versus oncogenic contexts might contribute to the development of new anti-Hdm2 drugs that are more efficient and less deleterious to healthy tissues, and thus limiting potential side effects of these promising anti-cancer therapies.

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### **Author contributions**

All the authors have approved the final version of the text. E. R. and O. B. have designed the study, have performed experiments, have analyzed and interpreted data, and have prepared the manuscript including text and figures. J. A. and D. S. have performed experiments, analyzed data, and participated in the preparation of the manuscript and figures. F. G., J. M., and M. H. have performed the training conditioning of the senior population at Montpellier University Hospital. T. G. has performed the training conditioning of the young male subjects at the Karolinska University Hospital. T. G. and T. L. H. have participated in the interpretation of data. All experiments on biopsies were carried out at the Angiogenesis Research Group at York University.

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## Figure legends

Figure 6.1 Exercise training increases expression of the endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1) in human skeletal muscle. (A and B) Representative immunoblots of PECAM-1 protein expression in the vastus lateralis muscle from young men (A, n = 16) or senior subjects (B, n = 14) before and after endurance training. C, Densitometric analysis of PECAM-1 protein expression is represented and  $\beta$ -actin was used as a loading control. Data are presented as means  $\pm$  SEM. The effect of exercise training or age was considered statistically significant when  $P \leq 0.05$  after two-way ANOVA analysis and Bonferroni posttest.

Figure 6.2 Exercise training increases expression of human double minute-2 (Hdm2) protein in human skeletal muscle. (A and B) Representative immunoblots of Hdm2 protein expression in the vastus lateralis muscle from young men (A, n = 16) or senior subjects (B, n = 14) before and after endurance training. C, Densitometric analysis of Hdm2 protein expression is represented and  $\beta$ -actin was used as a loading control. Data are presented as means  $\pm$  SEM. The effect of exercise training or age was considered statistically significant when  $P \leq 0.05$  after two-way ANOVA analysis and Bonferroni posttest. (D and E) Representation of individual responses to training for Hdm2 protein expression in muscles from young (D, n = 16) and senior subjects (E, n = 14). Individual responses are expressed as raw values (Hdm2 normalized to  $\beta$ -actin, top graph) and in percentage of change from pretraining (bottom graph). For each population, the percentages of Hdm2 responders (i.e., subjects having an increased expression of Hdm2 in response to training) and nonresponders are indicated.

Figure 6.3 Forkhead box O-1 (FoxO1) transcription factor expression is not affected by exercise training in human skeletal muscle. (A and B) Representative immunoblots of FoxO1 protein expression in the vastus lateralis muscle of young men (A, n = 16) or senior subjects (B, n = 7 men

and n = 7 women) before and after endurance training. Densitometric analysis of FoxO1 protein expression is represented and  $\beta$ -actin was used as a loading control. No significant difference was observed in response to exercise training (two-way ANOVA and Bonferroni posttests).



**Table 6.1****Table 1.** Clinical characteristics of the young and senior populations.

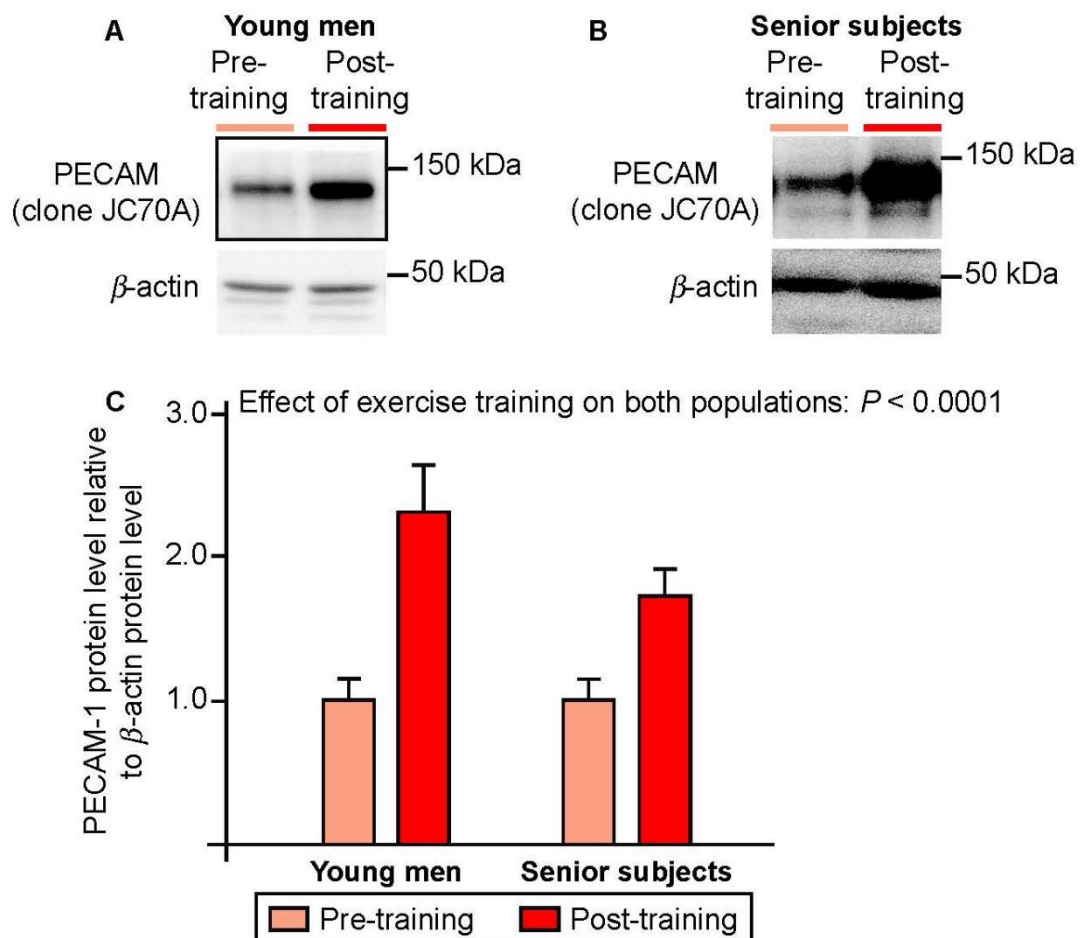
	Young men (n = 16)		Senior subjects (n = 14)	
Age (years)	23.4 ± 0.7		61.8 ± 1.7	
Height (cm)	179.7 ± 2.3		170.5 ± 1.9	
Body weight (kg)	73.9 ± 2.5		76.0 ± 3.0	
BMI (kg/m <sup>2</sup> )	22.9 ± 0.7		26.0 ± 0.7	
Peak VO <sub>2</sub> (mL min <sup>-1</sup> kg <sup>-1</sup> )	Pretraining	Posttraining*	Pretraining <sup>†</sup>	Posttraining* <sup>†</sup>
	49.7 ± 1.3	56.7 ± 1.8	25.7 ± 1.6	28.6 ± 1.6

Data are presented as means ± SEM. BMI, body mass index; Peak VO<sub>2</sub>, maximal oxygen consumption.

Statistical difference between pre- and posttraining conditions: \* $P \leq 0.001$ .

Differences between young and senior subjects: † $P \leq 0.01$ .

**Figure 6.1**



**Figure 6.2**

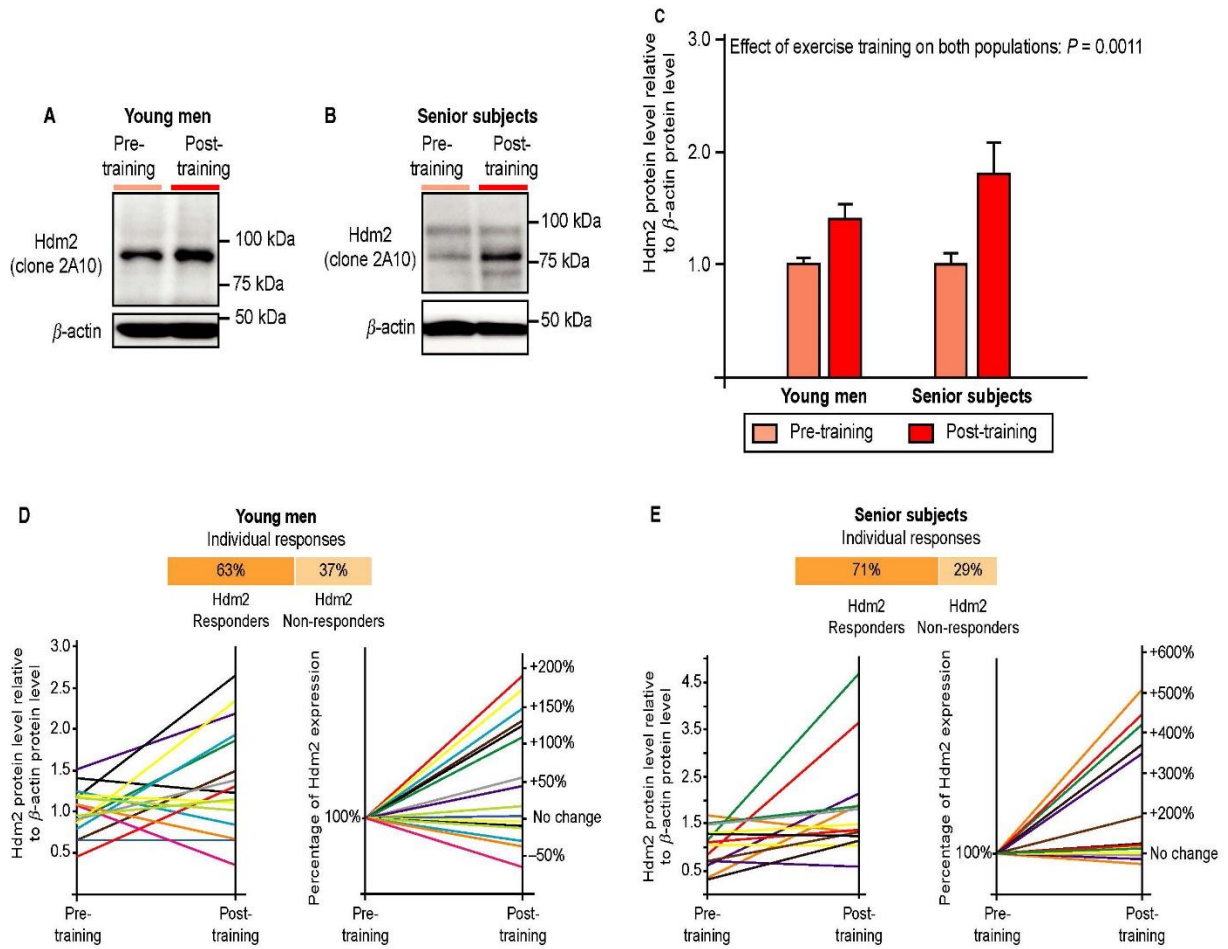
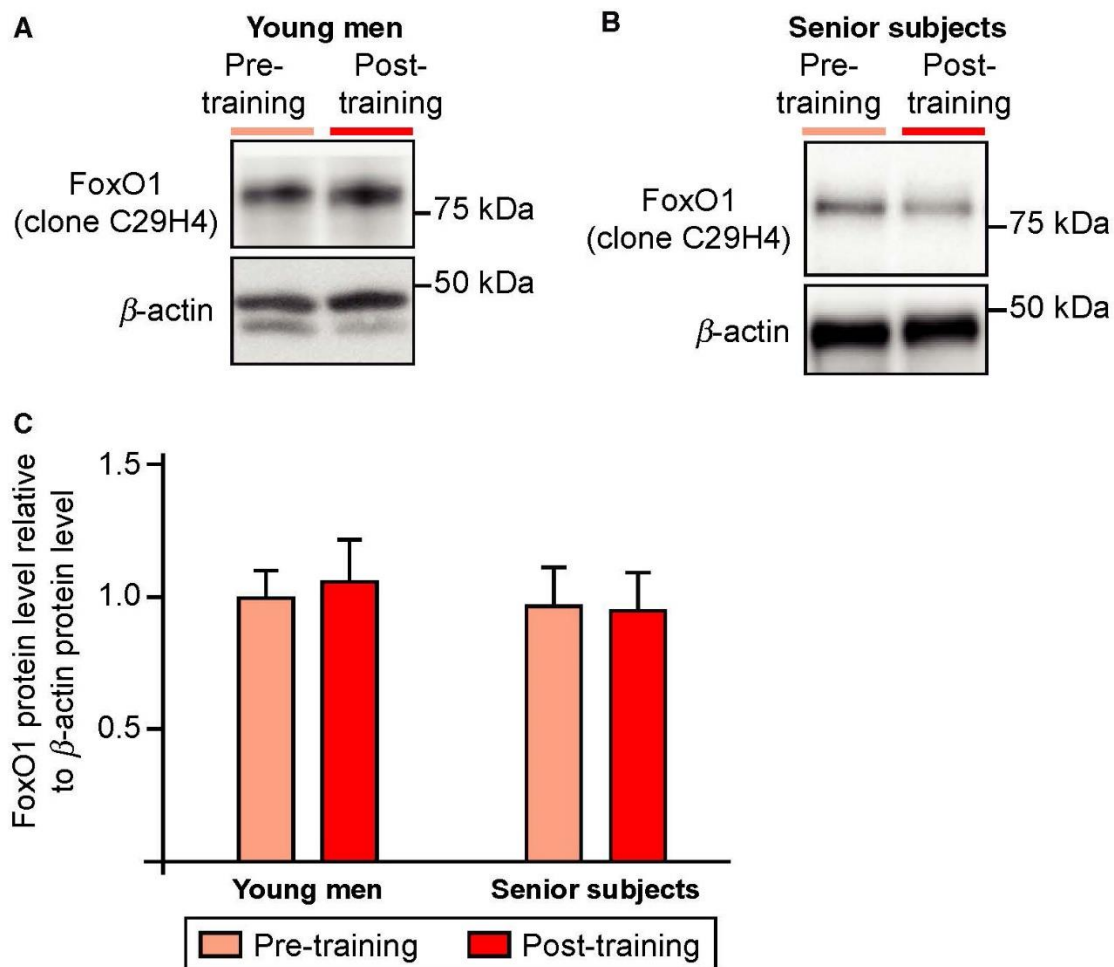


Figure 6.3



## 7. ACADEMIC RESEARCH PAPER 2

**Phosphorylation of murine double minute-2 on Ser166 is downstream of VEGF-A in exercised skeletal muscle and regulates primary endothelial cell migration and FoxO gene expression**

**Julian Aiken**, Emilie Roudier, Joseph Ciccone, Genevieve Drouin, Anna Stromberg, Jovana Vojnovic, I. Mark Olfert, Tara Haas, Thomas Gustafsson, Guillaume Grenier, and Olivier Birot

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### Author contributions

Julian Aiken:

- Contributed to formulate and design experiments
- Performed all experiments in Figures 4, 6, 7, 8 and 9
- Contributed to data analysis
- Contributed in the preparation of the manuscript and all figures

### Scientific context:

- We have shown that Mdm2 is essential for skeletal muscle capillary maintenance and for exercise-induced angiogenesis to occur
- Mdm2 protein is significantly elevated by exercise *training* in both rodent and human skeletal muscle

- In addition to understanding Mdm2 regulation in response to prolonged training, it is also important to study the role of Mdm2 during the ‘dynamic phase’ of the angiogenic process; *acute bouts* of exercise
- Mdm2 can undergo multi-site phosphorylation, and phosphorylation of Mdm2 on its serine 166 residue can lead to nuclear localization and an increased interaction with its downstream targets p53, FoxO1, and HIF-1 $\alpha$  [100,151,153,155,156].

**Research aims:**

- Investigate how an acute bout of exercise, representing the dynamic phase of angiogenesis, affects Mdm2 activity (i.e. phosphorylation)
- Investigate the impact of Mdm2 phosphorylation on endothelial cell pro-angiogenic activity
- Determine Mdm2-dependent molecular signaling that could regulate endothelial cell functionality

**Summary of main findings:**

- An acute bout of exercise strongly induces both VEGF-A and p-Ser166-Mdm2 protein
- VEGF-A has been identified as a potent stimulator of p-Ser166-Mdm2 in the muscle tissue and in endothelial cells
- VEGF-A-driven endothelial cell migration is dependent on Mdm2 activity
- Mdm2 phosphorylation on serine 166 leads to an increased interaction between Mdm2 and FoxO1 with subsequent down-regulation of FoxO1 target genes, namely, TSP-1 in primary human endothelial cells

- Our results show for the first time that Mdm2 is essential for the pro-angiogenic effect of VEGF-A, highlighting the complex and reciprocal relationship existing between the two molecules

**Phosphorylation of murine double minute-2 on Ser<sup>166</sup> is downstream of VEGF-A in exercised skeletal muscle and regulates primary endothelial cell migration and FoxO gene expression**

*FASEB J.* 2016 Mar;30(3):1120-34. doi: 10.1096/fj.15-276964

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Running head: Exercise-induced VEGF-A drives Mdm2 phosphorylation



## Abstract

We demonstrated in a previous study that murine double minute (Mdm)-2 is essential for exercise-induced skeletal muscle angiogenesis. In the current study, we investigated the mechanisms that regulate Mdm2 activity in response to acute exercise and identified VEGF-A as a key stimulator of Mdm2 phosphorylation on Ser166 (p-Ser166-Mdm2). VEGF-A and p-Ser166-Mdm2 protein levels were measured in human and rodent muscle biopsy specimens after 1 bout of exercise. VEGF-A-dependent Mdm2 phosphorylation was demonstrated *in vivo* in mice harboring myofiber-specific deletion of VEGF-A (mVEGF<sup>-/-</sup>) and *in vitro* in primary human and rodent endothelial cells (ECs). Exercise increased VEGF-A and p-Ser166-Mdm2 protein levels respectively by 157 and 68% in human muscle vs. pre-exercise levels. Similar results were observed in exercised rodent muscles compared to sedentary controls; however, exercise-induced Mdm2 phosphorylation was significantly attenuated in mVEGF<sup>-/-</sup> mice. Recombinant VEGF-A elevated p-Ser166-Mdm2 by 50–125% and stimulated migration by 33% in ECs when compared to untreated cells, whereas the Mdm2 antagonist Nutlin-3a abrogated VEGF-driven EC migration. Finally, overexpression of a Ser166-Mdm2 phosphorylation mimetic increased EC migration, increased Mdm2 to FoxO1 binding (+55%), and decreased FoxO1-dependent gene expression compared with ECs overexpressing WT-Mdm2. Our results suggest that VEGF-mediated Mdm2 phosphorylation on Ser166 is a novel proangiogenic pathway within the skeletal muscle.

## **Introduction**

Angiogenesis is the formation of new capillaries from pre-existing ones, a process that can be observed under various physiologic and pathologic circumstances (1). Endurance training is a well-established illustration of physiologic angiogenesis in adult skeletal muscle tissue (2). A single bout of exercise represents a strong physiologic stress for the skeletal muscle, modulating the expression of pro- and antiangiogenic molecules and inducing significant changes in gene expression. If this exercise stimulus is repeated persistently [i.e., during daily exercise training, the balance between pro- and antiangiogenic factors is shifted toward a proangiogenic profile (3)], ultimately, endothelial cells will migrate and assemble into new capillaries. This expansion of the microvascular bed ensures that oxygen and nutrient supply is well matched with the metabolic needs of active myofibers (2). Several key factors have been identified as important regulators of angiogenesis in the adult skeletal muscle, particularly the proangiogenic VEGF-A and the antiangiogenic thrombospondin (TSP)-1 (3).

In this context, we recently demonstrated that the E3 ubiquitin ligase murine double minute (Mdm)-2 is a critical regulator of capillary maintenance and exercise-induced angiogenesis in skeletal muscles (4). Using transgenic mice with reduced Mdm2 expression, we demonstrated that Mdm2 is essential for exercise-induced VEGF-A expression, endothelial cell migration, and training-induced muscle angiogenesis (4).

Mdm2 was studied primarily for its role as a negative regulator of the tumor suppressor p53, and it may affect angiogenesis by inhibiting p53-dependent antiangiogenic activities, such as induction of TSP-1 expression. It also may exert proangiogenic activity independently of p53 (5). Mice that overexpress Mdm2 have a higher incidence of angiosarcoma, a type of tumor characterized by an excessive proliferation of endothelial cells (ECs), independent of their p53

status (6). In line with this knowledge, we have shown that reduced Mdm2 expression in skeletal muscle results in lower levels of capillarization independent of p53 expression (4), whereas p53 knockout without concomitant alteration of Mdm2 expression has no effect on muscle capillarization (7). Aside from p53, Mdm2 interacts with other transcription factors that are essential for skeletal muscle angiogenesis, such as hypoxia inducible factor (HIF)-1 $\alpha$  and forkhead box O1 (FoxO1). Recent in vitro studies indicated that Mdm2, when phosphorylated on Ser166, stimulates VEGF-A expression through HIF-1 $\alpha$  stabilization (8, 9). Most of these studies were performed on tumor cells where Mdm2 is amplified or overexpressed, and the physiologic relevance of such regulatory effects of Mdm2 on VEGF-A expression remains uncertain. Mdm2 can sense endogenous and exogenous stresses through posttranslational modifications, such as site-specific phosphorylation, that in turn control Mdm2 activity and recruitment of downstream targets (5, 10). For example, phosphorylation on Ser166 in response to insulin-like growth factor-1 promotes degradation of p53 and HIF-1  $\alpha$  stabilization in tumor cells (11). Mdm2 also binds to FoxO proteins (12). In recent studies, we demonstrated that endothelial FoxO transcription factors promote an antiangiogenic microenvironment in skeletal muscle in both pathologic and physiologic contexts (13, 14). We have shown that mice harboring an EC-directed deletion of FoxO exhibit an earlier onset of exercise-induced angiogenesis than do their wild-type (WT) littermates (14). In another study, we showed that phosphorylated Mdm2 on Ser166 interacts with FoxO1 in the skeletal muscle and in endothelial cells (12), suggesting that Mdm2 negatively regulates FoxO activity.

In the present study, we investigated further how one bout of exercise regulates Mdm2 activity in skeletal muscle. In our work, in the physiologic context of exercise, VEGF-A was an upstream regulator of Mdm2 phosphorylation on Ser166 (p-Ser166-Mdm2). Taking into

consideration that other studies have positioned Mdm2 as an upstream regulator of VEGF-A expression, we now bring evidence of a more complex relationship between the 2 molecules. We found that Mdm2 activity was necessary for EC migration in response to exercise and VEGF-A stimulation. Overexpression of a phosphomimetic form of p-Ser166-Mdm2 (S166D-Mdm2) promoted EC migration, increased the binding between Mdm2 and FoxO1, and decreased gene expression of hallmark FoxO targets in primary ECs. Altogether, our results suggested that Mdm2 activation by phosphorylation on Ser166 and its subsequent impact on FoxO1 targets represent a novel proangiogenic signaling pathway downstream of VEGF-A in exercised human and rodent skeletal muscle.

## **Materials and methods**

*Exercise protocol and muscle biopsies from human subjects.* Experiments were performed after informed consent was obtained from the subjects and after ethics approval (Karolinska Institutet 2006/1232-31/1). Five healthy male subjects were included in the study. Their mean (range) age, height, and weight were 27 (22–34) yr, 180 (173–187) cm, and 75 (69–83) kg, respectively. They were moderately active, which corresponded to a mean (range) maximum oxygen consumption ( $VO_{2max}$ ) of 48 (41–57) ml/kg/min. The subjects had no history of chronic disease. They performed 60 min of cycle exercise on an electrodynamically loaded cycle ergometer. During the first 20min, they cycled at 60 rpm at a median (range) work rate of 125 (105–145) W, chosen to correspond to 50% of  $VO_{2max}$ , after which the work rate was increased to 170 (140–190) W, corresponding to 65% of  $VO_{2max}$  for a further 40min. Blood pressure and heart rate were measured every 10min during exercise. Muscle biopsy samples were obtained by the percutaneous needle biopsy technique from the vastus lateralis muscle at rest, immediately after 60 min of exercise, and after 120 min of recovery after exercise. All biopsy samples were frozen in liquid nitrogen until analysis.

*Exercise protocols in rodents.* Animal experiments were conducted according to the Canadian Council on Animal Care, and the animal care and use committees of York University and West Virginia University. Nine week-old female Sprague-Dawley rats (n = 24; 180–200 g) were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). Engineering of myofiber-specific VEGF-A deficient mice (mVEGF<sup>-/-</sup>) was as described elsewhere (15).

After familiarization to treadmill running, the animals performed a single bout of running exercise on a rodent treadmill until exhaustion (4, 15). Plantaris or gastrocnemius muscles were collected immediately after exercise. The rats performed a running exercise at 25–30 m/min, at a 10% incline, for 60–90 min (n = 8 rats per group). WT and mVEGF<sup>-/-</sup> mice ran on the treadmill at 10 m/min for 10 min with a 10% incline, followed by 15 m/min until exhaustion (18–20 min), as described previously (15).

*Three-dimensional muscle explant assay.* The 3-dimensional (3D) muscle explant assay was performed according to a published method (4, 13). Muscle explants were embedded in type-1 collagen. In some wells, the Mdm2 inhibitor Nutlin-3a was added to the gel and medium with 2% fetal calf serum (FCS; 1 mM; N6287; Sigma-Aldrich, Oakville, ON, Canada). After 6 d, the 3D muscle explants were fixed with 4% formalin, and endothelial outgrowth was detected with isolectin-B4 and tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (cat. no. 016-020-084; Jackson ImmunoResearch, West Grove, PA, USA). Images were acquired with an inverted microscope. The relative number of ECs was quantified by measuring the intensity of staining of the major migration front surrounding the muscle biopsy. The value attributed to an animal is the average of 4–6 analyzed biopsy specimens. Data are represented as staining intensity (arbitrary units) per pixel (n = 4 animals per group).

*In vitro plantaris biopsy sample incubation.* Forty milligrams of muscle were rinsed 3 times in PBS and DMEM before incubation in DMEM with 2% FCS and stimulation with increasing concentrations of recombinant VEGF<sub>165</sub> (recVEGF<sub>165</sub>; 0–50 ng/ml; Peprotech, Rocky Hills, NJ, USA), or 10% muscle homogenates prepared from plantaris muscles of sedentary and exercised rats for 2 h. After muscle lysis with a MM400 tissue lyser (30 pulses/s at 4°C; Retsch GmbH, Haan, Germany), they were centrifuged at 4°C for 15 min at 15,000 g, and the supernatants were collected to serve as homogenates. Before muscle homogenate stimulation, biopsies specimens were preincubated or not with 200 nM of the VEGF-A receptor inhibitor KRN633 (sc-204379; Santa Cruz Biotechnology, Dallas, TX, USA).

*Cell culture.* Rat or mouse skeletal muscle microvascular ECs (SMECs) were isolated from extensor digitorum longus and tibialis anterior muscles and cultured as has been described (12, 13). The cells were starved overnight with DMEM containing 1% FCS before stimulation with recombinant rat or mouse VEGF<sub>165</sub>, as indicated in the figure legends (Peprotech).

Primary human dermal and human adipose microvascular ECs (HDMECs and HAMECs, respectively) were purchased from ScienCell Research Laboratories (cat. no. 2000 and 7200, respectively; Carlsbad, CA, USA). The cells were maintained in ECM (cat. no. 1001) supplemented with 5% FBS (cat. no. 0025), 1% EC growth supplement (ECGS; cat. no. 1052), and antibiotic solution containing 100 U/ml penicillin and 100 µg/ml streptomycin (cat. no. 0503; all from ScienCell). HAMECs and HDMECs were starved with ECM containing 1% FBS before stimulation with recombinant human VEGF<sub>165</sub> (Peprotech).

*Generation of WT-Mdm2 and S166D-Mdm2 mutant cell lines.* Lentiviruses encoding WT-Mdm2 (cat. no. 16233; Addgene) or mutant S166D-Mdm2 (cat. no. 16234; Addgene) plasmids were generated by subcloning into lentiviral vector with puromycin selection, pLenti CMV/TO Puro

DEST (cat. no. 17293; Addgene) (16), and viral particles were produced by transfecting 293T cells with the packaging vectors (pLP1, pLP2, and pLP/VSVG) (ViraPower; Thermo Scientific-Invitrogen) and pLenti Mdm2 or pLenti S116DMdm2. Subconfluent HDMECs in culture were infected with one of the lentiviruses. Transduced cells were selected with 2  $\mu$ g/ml puromycin and subcultured in endothelial cell medium (ECM) with 1  $\mu$ g/ml puromycin until use in experiments, generating WT-Mdm2 and mutant S166-Mdm2 (S166D-Mdm2)–overexpressing HDMECs.

*Immunoblot analysis.* Immunoblot analysis was conducted on protein extracts from rodent plantaris or gastrocnemius muscles, human vastus lateralis biopsies, or primary ECs (4, 13). Blots were probed with the following primary antibodies:  $\alpha$  $\beta$ -tubulin (cat.no. 2148; Cell Signaling Technology, Beverly, MA, USA), Mdm2 clone SMP14 (sc-965; Santa Cruz Biotechnology), Mdm2 clone 2A10 (4), Mdm2 4B11 (Ab-3; Calbiochem, Etobicoke, ON, Canada), p-Ser166-Mdm2 (cat.no. 3521; Cell Signaling Technology),  $\beta$ -actin (sc-47778; Santa Cruz Biotechnology), VEGF-A (VG-1; Millipore, Etobicoke, ON, Canada), p53 (sc-6243; Santa Cruz Biotechnology), HIF-1 $\alpha$  (NB100-105; Novus Biologicals, Littleton, CO, USA), and FoxO1 (C29H4, catno. 2880; Cell Signaling Technology). After incubation with secondary antibody (horseradish peroxidase [HRP]-linked anti-rabbit antibody, cat. no. 7074, Cell Signaling Technology; or HRP-linked anti-mouse; cat. no. P0260; Dako, Carpinteria, CA, USA), proteins were visualized with enhanced chemiluminescence (Santa Cruz Biotechnology) on Imaging Station 4000MM Pro (Carestream Health, Rochester, NY, USA). Blots were analyzed with Image 1.62 (National Institutes of Health, Bethesda, MD, USA) or Carestream software.

*Immunoprecipitation.* HDMECs overexpressing WT or S166D-Mdm2 were treated with 20  $\mu$ M MG132 (I-130; Boston Biochem, Cambridge, MA, USA) for 5 h before cell lysis, to block proteasomal degradation. FoxO1, Mdm2, or p53 were immunoprecipitated by overnight

incubation with the respective primary antibodies (cat. no. 2880; Cell Signaling Technology, and sc-965 and sc-126; Santa Cruz Biotechnology) and Immunocruz Optima F IP matrix (sc-45043; Santa Cruz Biotechnology). Normal rabbit (sc-2027) or mouse (sc-2025; both from Santa Cruz Biotechnology) IgG was used as a species-specific control primary antibody. Cell lysates were precleared (Immunocruz Optima F preclearing matrix; sc-45060; Santa Cruz Biotechnology) and incubated overnight with the IP antibody–matrix complex. Supernatants were analyzed by Western blot for FoxO1 (Cell Signaling Technology), Mdm2 (clone 4B11, OP143 or non-commercial 2A10; Calbiochem), p53 (sc-6243; Santa Cruz Biotechnology), or HIF-1 $\alpha$  (cat. no. 100-105; Novus Biologicals).

*Boyden chamber migration assay.* Assessment of EC migration was performed with HDMECs in the Boyden chamber assay (17). HDMECs were pretreated with the Mdm2 inhibitor Nutlin-3a (10  $\mu$ M) or vehicle for 1 h before trypsinization and resuspension in ECM with 1% FBS at a concentration of 400 cells/ml. Cell migration was stimulated by recVEGF<sub>165</sub> (100 ng/ml). Cells migrated through an 8- $\mu$ m polycarbonate membrane filter (cat. no. PFB8; Neuro Probe, Inc., Gaithersburg, MD, USA) coated with 50 $\mu$ g/ml collagen (cat. no. A10438-01; Thermo Scientific-Gibco, Grand Island, NY, USA) in 0.02 M acetic acid (cat. no. 320099; Sigma-Aldrich, St. Louis, MO, USA). After 4.5 h of migration time, the membrane filter was fixed in cold methanol, stained with 10% Giemsa stain diluted in water (cat. no. GS500; Sigma-Aldrich), and mounted on a microscope slide. Three independent experiments were performed with 4–6 wells per condition (with or without recVEGF<sub>165</sub> and with or without Nutlin-3a).

To assess the role of p-Ser166-Mdm2 phosphorylation on HDMECs migration, we performed the Boyden chamber assay on HDMECs infected with lentiviruses encoding for either WT-Mdm2 or S166D-Mdm2. The bottom chambers were loaded with 28  $\mu$ l of either ECM with



1% FBS and no ECGS (no-stimulation condition) or ECM supplemented with 10% FBS and 5% ECGS (stimulation condition). Two separate experiments were performed with 4–6 wells per condition per experiment.

For each experiment, 4 individual fields of view per well were counted under X40 magnification. Results are presented as the average of all fields of view.

*Statistical analysis.* Statistical analyses were performed with Student's t test and 1- and 2-way ANOVAs with Prism5 (GraphPad, San Diego, CA, USA). For 1- and 2-way ANOVAs, Newman-Keuls multiple comparison and Bonferroni post hoc tests were used, respectively. Correlation analyses between variables were performed with nonparametric 2-tailed Pearson correlation with determination of Pearson  $r$  and  $r^2$ .  $P \leq 0.05$  was considered to be statistically significant.

## **Results**

*Exercise stimulates VEGF-A expression in human and rodent skeletal muscles.* Whereas a single bout of exercise had no effect on the Mdm2 protein level in human (Fig. 7.1A), rat (Fig. 7.1C), and mouse skeletal muscles (Fig. 7.2E, G), it strongly stimulated p-Ser166-Mdm2 (Figs. 7.1A–D and 7.2E, F). The p-Ser166-Mdm2 level increased significantly (68%) in human (postexercise vs. pre-exercise,  $0.61 \pm 0.07$  vs.  $0.27 \pm 0.18$ ;  $P \leq 0.05$ ) and by 128% in rat skeletal muscles (postexercise vs. pre-exercise,  $0.57 \pm 0.09$  vs.  $0.25 \pm 0.05$ ;  $P \leq 0.05$ ). This phosphorylation was transient, however, as no significant increase was detected at 2 and 4 h after exercise in human or rat muscles, respectively (Fig. 7.1A–D). VEGF-A protein in response to 1 bout of exercise followed a pattern similar to that of p-Ser166-Mdm2 levels. VEGF-A protein levels were increased by 157% in exercised human biopsy samples (postexercise vs. pre-exercise,  $0.90 \pm 0.18$  vs.  $0.35 \pm 0.11$ ;  $P \leq 0.05$ ) and by 45% in rat muscles (postexercise vs. preexercise,  $0.46 \pm 0.03$  vs.  $0.30 \pm$

0.02;  $P \leq 0.05$ ). At 2 h after exercise, no significant increase in VEGF-A was observed in human muscles and the increase in rat samples, although still significant, was considerably attenuated. The transient increase in p-Ser166-Mdm2 correlated positively with the increase in VEGF-A protein levels in human skeletal muscle (Fig. 7.1E;  $r^2 = 0.621$ ;  $P \leq 0.0005$ ). Similar data were recorded in rat skeletal muscle (data not shown; correlation slope =  $0.062 \pm 0.01$ ;  $r^2 = 0.879$ ;  $P \leq 0.001$ ).

*Muscle VEGF-A expression is essential for Mdm2 phosphorylation in response to exercise.*

In transgenic mice harboring a muscle-specific deletion of VEGF-A (mVEGF<sup>-/-</sup>), we investigated whether Mdm2 phosphorylation on Ser166 is dependent on exercise-induced expression of VEGF-A. As has been described (15), mVEGF<sup>-/-</sup> mice expressed less VEGF-A protein in their skeletal muscle at rest (Fig. 7.2A) and presented a severe reduction (257%) in their level of muscle capillarization (Fig. 7.2B). In contrast to their WT littermates, no significant increase in VEGF-A was observed in the mVEGF<sup>-/-</sup> mice after one bout of running exercise (Fig. 7.2C, D; WT,  $0.28 \pm 0.09$  at rest vs.  $0.57 \pm 0.03$  after exercise;  $P \leq 0.05$ ; mVEGF<sup>-/-</sup>,  $0.18 \pm 0.03$  at rest vs.  $0.30 \pm 0.05$  after exercise;  $P > 0.05$ ). Whereas mVEGF<sup>-/-</sup> mice expressed slightly more Mdm2 protein than did WT animals at basal levels (Fig. 7.2E, F; 2-way ANOVA, overall effect of genotype,  $P = 0.0092$ ), no change in Mdm2 protein level was observed after one bout of running exercise in both WT and mVEGF<sup>-/-</sup> mice (Fig. 7.2E, F). p-Ser166-Mdm2 protein levels were strongly increased by exercise in WT animals (Fig. 7.2E, G; +71%;  $0.14 \pm 0.03$  at rest vs.  $0.24 \pm 0.02$  after exercise;  $P \leq 0.05$ ) but no significant increase was observed in mVEGF<sup>-/-</sup> mice (Fig. 7.2E, G;  $0.10 \pm 0.02$  at rest vs.  $0.18 \pm 0.03$  after exercise;  $P > 0.05$ ). Given the effect of the genotype on total Mdm2 expression, we analyzed the p-Ser166-Mdm2:Mdm2 ratio (Fig. 7.2H). Whereas this ratio increased in WT mice with exercise (+77%;  $0.49 \pm 0.05$  at rest vs.  $0.87 \pm 0.07$  after exercise;  $P \leq 0.05$ ), no

significant change was detected in mVEGF<sup>-/-</sup> mice (+24%;  $0.33 \pm 0.05$  at rest vs.  $0.41 \pm 0.08$  after exercise;  $P > 0.05$ ). VEGF-A deletion in muscle cells therefore strongly reduced the exercise-induced increase in p-Ser166-Mdm2. This finding suggests that VEGF-A is an upstream regulator of Mdm2 phosphorylation on Ser166 in exercised skeletal muscle.

*VEGF-A stimulation promotes the phosphorylation of Mdm2 on Ser166 in ex vivo skeletal muscle biopsies.* To confirm that VEGF-A could act as an upstream regulator of Mdm2 phosphorylation, plantaris biopsy samples from sedentary rats were incubated in vitro in the presence of increasing concentrations of recVEGF<sub>165</sub> (0–50 ng/ml) for 2 h (Fig. 7.3A, B). VEGF-A stimulated an increase in p-Ser166-Mdm2 protein levels (1-way ANOVA;  $P < 0.0001$ ). Next, some samples from sedentary rats were incubated in vitro in the presence of 10% muscle homogenate obtained from sedentary or exercised rats. Whereas the stimulation with the exercise homogenate significantly induced p-Ser166-Mdm2 levels in the plantaris specimen (Fig. 7.3C;  $0.22 \pm 0.02$  sedentary homogenate vs.  $0.30 \pm 0.02$  exercise homogenate; 2-way ANOVA;  $P \leq 0.05$ ), this effect was abolished in the presence of the VEGF receptor inhibitor KRN933 (Fig. 7.3D;  $0.25 \pm 0.02$  sedentary homogenate with KRN633 vs.  $0.26 \pm 0.04$  exercise homogenate with KRN633).

*VEGF-A stimulates Mdm2 phosphorylation on Ser166 in primary ECs.* Both exercise and VEGF-A stimulated the phosphorylation of Mdm2 on Ser166 in skeletal muscle. Therefore, we sought to determine whether Ser166 phosphorylation occurs more specifically in the endothelium of the skeletal muscle.

We incubated primary microvascular ECs isolated from rat skeletal muscles (rSMECs) with increasing doses of rat recVEGF<sub>165</sub> (0–100 ng/ml) for 1 h. VEGF-A increased p-Ser166-Mdm2 in rSMECs (Fig. 7.4A). We further studied the effect of VEGF-A stimulation on primary

microvascular ECs from mSMECs, as well as HAMECs and HDMECs (Fig. 7.4B). One hour of stimulation with recVEGF<sub>165</sub> increased the levels of p-Ser166-Mdm2 by 50, 108, and 125% in mSMECs, HAMECs, and HDMECs, respectively (recVEGF<sub>165</sub> vs. control: mSMECs,  $1.51 \pm 0.13$  vs.  $1.00 \pm 0.05$ ;  $P = 0.0234$ ; HAMECs,  $2.08 \pm 0.28$  vs.  $1.00 \pm 0.04$ ;  $P = 0.0178$ ; and HDMECs,  $2.25 \pm 0.5$  vs.  $1.00 \pm 0.09$ ;  $P = 0.0286$ ). No significant change in the level of Mdm2 protein expression was observed in response to recVEGF<sub>165</sub> stimulation.

*Mdm2 inhibitor Nutlin-3a inhibits exercise-induced endothelial outgrowth from skeletal muscle explants.* To test the hypothesis that Mdm2 activity is necessary for SMEC migration in the context of exercise, we performed an ex vivo skeletal muscle explant assay by embedding plantaris biopsy specimens from sedentary and exercised rats in a 3D collagen gel in the presence of Nutlin-3a (Fig. 7.5A). Biopsy samples were obtained from the plantaris muscles as presented in Fig. 7.1, where postexercise levels of VEGF-A and p-Ser166-Mdm2 protein are both shown to have increased. A single bout of running exercise strongly increased endothelial outgrowth compared with the sedentary condition (Fig. 7.5B, C) (+118%;  $34.8 \pm 2.4$  at rest vs.  $76.00 \pm 7.6$  after exercise; 2-way ANOVA;  $P \leq 0.0001$ ). However, this stimulatory effect was abolished in the presence of Nutlin-3a ( $24.4 \pm 1.6$  at rest vs.  $22.8 \pm 3.3$  after exercise) (Fig. 7.5C, D). No significant effect of Nutlin-3a was observed under resting conditions. These results suggest that Mdm2 activity is necessary for exercise-induced EC migration in skeletal muscle.

*Mdm2 inhibitor Nutlin-3a inhibits VEGF-A-induced migration of primary microvascular ECs.* Next, we wanted to know whether Mdm2 activity is specifically essential for VEGF-A-induced EC migration (Fig. 7.6). Incubation with human recVEGF<sub>165</sub> of HDMECs used in the Boyden chamber migration assay efficiently stimulated the phosphorylation of Mdm2 on Ser166 (Fig. 7.6A; no VEGF-A,  $1.00 \pm 0.12$  vs. VEGF-A treated,  $1.83 \pm 0.13$ ;  $P \leq 0.01$ ), whereas total

Mdm2 protein levels were unaltered (no VEGF-A,  $1.00 \pm 0.11$  vs. VEGF-A treated,  $1.03 \pm 0.07$ ). These data served as a control to confirm that the ECs subsequently placed into the migration chamber exhibited an elevated level of p-Ser166-Mdm2 after recVEGF<sub>165</sub> incubation. Endothelial cell migration was significantly enhanced (+33%) with VEGF-A stimulation (Fig. 7.6B; untreated,  $100.0 \pm 1.4\%$  vs. VEGF-A stimulation,  $133.0 \pm 2.1\%$ ;  $P \leq 0.001$ ). However, Nutlin-3a fully abolished VEGF-A's stimulatory effects (untreated,  $100.0 \pm 1.4\%$  vs. VEGF-A + Nutlin-3a,  $102.0 \pm 2.0\%$ ;  $P > 0.05$ ). Nutlin-3a treatment had no significant effect on basal EC migration (untreated,  $100 \pm 1.4\%$  vs. Nutlin-3a,  $96.4 \pm 1.3\%$ ;  $P > 0.05$ ).

In addition, stimulation of HDMECs with 10% FBS and 5% ECGS was used as a standard positive control for the Boyden chamber assay, showing an 80% increase in EC migration (Fig. 7.6C; untreated,  $100.0 \pm 0.1\%$  vs. FBS +ECGS,  $181.0 \pm 2.5\%$ ;  $P \leq 0.001$ ). Nutlin-3a partially inhibited (~14%) the FBS/ECGS-induced migration of ECs (FBS/ECGS,  $181.0 \pm 2.5\%$  vs. FBS/ECGS + Nutlin-3a,  $155.0 \pm 2.0\%$ ).

*Mdm2 phosphorylation on Ser166 regulates EC migration.* To specifically assess the functional impact of p-Ser166-Mdm2, we generated HDMECs that overexpressed WT-Mdm2 or S166D-Mdm2. Both WT-Mdm2 and S166D-Mdm2 showed increased protein expression levels of Mdm2 vs. levels in noninfected HDMECs (Fig. 7.7A, B; noninfected,  $6.32 \pm 0.36$  vs. WT-Mdm2,  $24.09 \pm 1.90$ ; +281%;  $P \leq 0.001$  and vs. S166D-Mdm2,  $18.41 \pm 1.24$ ; +191%;  $P \leq 0.001$ ). As expected, the S166D-Mdm2 showed a loss of reactivity to the anti-p-Ser-166-Mdm2 antibody detection (Fig. 7.7C). The migratory activity of infected HDMECs was evaluated in the Boyden chamber assay (Fig. 7.7D, E). Overexpression of WT-Mdm2 did not affect basal HDMEC migration (noninfected HDMECs  $100.0 \pm 2.3\%$  vs. WT-Mdm2  $95.0 \pm 3.4\%$ ;  $P > 0.05$ ). In contrast, overexpression of S166D-Mdm2 significantly enhanced (+38%) the basal migration level (without

any source of stimulation) (noninfected HDMECs  $100.0 \pm 2.3\%$  vs. S166D-Mdm2  $138.0 \pm 8.4\%$ ;  $P \leq 0.001$ ). Stimulation with 10% FBS + 5% ECGS enhanced migration in all cell lines by  $95 \pm 8.76\%$  for non-infected HDMECs,  $116 \pm 4.7638\%$  for WT-Mdm2, and  $275 \pm 4.17\%$  for S166D-Mdm2, compared with each corresponding nonstimulated condition ( $P \leq 0.001$ ). The percentage of HDMECs migrating under FBS + ECGS stimulation was significantly higher in the S166D-Mdm2 cells when compared with the percentages of both the stimulated noninfected and WT-Mdm2 cells ( $P \leq 0.001$ ). This result suggests that Mdm2 phosphorylation on Ser166 is essential for basal and stimulated primary microvascular EC migration.

*Ser166 phosphorylation enhances Mdm2 binding to FoxO1 and leads to the down-regulation of FoxO target genes.* We have shown that the level of p-Ser166-Mdm2 correlates negatively with FoxO1 levels (12). In the current study, we investigated whether Mdm2 phosphorylation on Ser166 changes the ability of Mdm2 to bind FoxO1 in WT-Mdm2 and S166D-Mdm2 cells. The anti-FoxO1 antibody pulled down more Mdm2 in S166D-Mdm2 cells (Fig. 7.8A). The ratio between the levels of Mdm2 and FoxO1 that were coimmunoprecipitated was higher in S166D-Mdm2 than in WT-Mdm2 HDMECs ( $0.31 \pm 0.02$  vs.  $0.16 \pm 0.02$ , respectively). This finding suggests ~91% more binding between Mdm2 and FoxO1 in ECs overexpressing the S166D-Mdm2. No differences were observed in Mdm2-p53 or Mdm2-HIF-1 $\alpha$  binding, respectively, between WT-Mdm2- and S166D-Mdm2-overexpressing cells (Supplemental Fig. 7.1).

Next, we measured the level of expression of p27, Sprouty-2, and TSP-1mRNAs, all regulated by FoxO in ECs (13, 18). mRNA levels of all FoxO target genes decreased significantly in S166D-Mdm2 vs. WT-Mdm2 cells (Fig. 7.8B–D): p27 mRNA, -34%;  $0.92 \pm 0.07$  in WT-Mdm2 vs.  $0.64 \pm 0.03$  in S166D-Mdm2;  $P = 0.037$ ; TSP-1 mRNA, -46%;  $1.20 \pm 0.05$  in WT-Mdm2 vs.

0.65 ± 0.07 in S166D-Mdm2; P ≤ 0.0001; and Sprouty-2 mRNA, -26%; 1.18 ± 0.02 in WT-Mdm2 vs. 0.87 ± 0.08 in S166D-Mdm2; P = 0.047.

We also assessed the effect of S166D-Mdm2 overexpression on VEGF-A mRNA levels. VEGF-A is a strong regulator of angiogenesis that can be regulated by Mdm2 (10) and FoxO1 (19, 20). S166D-Mdm2 cells expressed reduced levels of VEGF-A mRNA compared with WT-Mdm2 HDMECs (Fig. 7.8E; -20%; WT-Mdm2, 1.17 ± 0.02 vs. S166D-Mdm2, 0.94 ± 0.06; P ≤ 0.006).

We have described the VEGF-A:TSP-1 ratio to be a good indicator of the angiogenic balance in skeletal muscle (3, 4). This ratio increased by 56% in S166D-Mdm2 vs. WT-Mdm2 cells (Fig. 7.8F; S166-Mdm2, 1.53 ± 0.130 vs. WT-Mdm2, 0.98 ± 0.04; P ≤ 0.028) suggesting a shift of the angioadaptive balance toward a more proangiogenic profile in S166D-Mdm2 ECs.

*VEGF-A stimulation affects FoxO target genes and the VEGF-A/TSP-1 ratio in primary human ECs.* The time-course stimulation of primary HDMECs with recVEGF<sub>165</sub> (100 ng/ml) led to decreased mRNA expression of the FoxO target genes p27 and TSP-1 (Fig. 7.9A, B). p27mRNA expression was affected rapidly (effect of time, P = 0.0039) with a significant decrease at all of the time points (Fig. 7.9A; no stimulation (0 h), 1.39 ± 0.18 vs. 1 h, 0.99 ± 0.05; P ≤ 0.05; 3 h, 0.68 ± 0.05; P ≤ 0.01; and 6 h, 0.63 ± 0.07; P ≤ 0.01). A decrease in TSP-1 mRNA expression was also observed (effect of time, P = 0.0099), becoming significant at 6 h (Fig. 7.9B; -48%; no stimulation (0 h), 1.14 ± 0.07 vs. 6 h, 0.55 ± 0.04; P ≤ 0.01).

The VEGF-A mRNA level was significantly up-regulated with recVEGF<sub>165</sub> stimulation at all time-points (Fig. 7.9C; 1-way ANOVA overall effect, P = 0.0109; no stimulation (0h), 0.71 ± 0.13 vs. 1 h (+110%), 1.49 ± 0.13, 3 h (+94%), 1.40 ± 0.01, and 6 h (+62%), 1.15 ± 0.05; significantly different from (0 h), P ≤ 0.05). Combined, the increase in VEGF-A and the decrease

in TSP-1 resulted in an increased VEGF-A:TSP-1 ratio that became significant at 6h (Fig. 7.9D; +200%; no stimulation (0 h),  $0.64 \pm 0.09$  vs. 6h,  $1.93 \pm 0.11$ ;  $P = 0.006$ ).

## **Discussion**

In our study, Mdm2 activation by phosphorylation on Ser166 occurred as a robust response of the skeletal muscle to acute physical exercise, observable both in rodent and human skeletal muscle. Mdm2 phosphorylation was regulated by VEGF-A and had a strong functional impact on EC migration. The evidence showed that Mdm2 phosphorylation on Ser166 modulated the angioadaptive balance, in particular the ratio between VEGF-A and TSP-1, by interacting directly with the transcription factor FoxO1. Altogether, our results identified Mdm2 as a novel downstream effector of VEGF-A in the physiologic context of exercise-induced muscle angiogenesis.

A single bout of exercise increases muscle expression and secretion of VEGF-A, as we and others have reported (4,21). This effect should increase VEGFR-2 activation on ECs and its downstream signaling, including several kinases (22), which in turn are known to regulate Mdm2 Ser166 phosphorylation and its enzymatic activity (10). In our study, muscle-derived VEGF-A was a key regulator of Mdm2 Ser166 phosphorylation. Not only did the exercise-induced increase in Ser166 phosphorylation of Mdm2 closely match the pattern of change in VEGF-A protein in both human and rodent muscles, but this effect was also absent in mice deficient in myocyte-VEGF-A. Direct stimulation of cultured ECs with recombinant VEGF-A provided additional evidence that Ser166 phosphorylation of Mdm2 is a downstream consequence of VEGF receptor activation.



Prior work has identified Mdm2 as an upstream regulator of VEGF-A expression in various cell lines (23). We recently identified Mdm2 as a critical regulator of skeletal muscle angioadaptation, both at rest and during exercise. Exercise-induced VEGF-A expression in skeletal muscle was strongly attenuated in mice harboring a reduced expression of Mdm2, compared to their exercised littermate controls (4). This effect occurred independent of the animals' p53 status, supporting the concept that Mdm2 could contribute to microvascular homeostasis in a p53-independent manner and through the regulation of other transcription factors.

Mdm2 can indeed regulate the activity of other transcription factors, such as HIF-1 $\alpha$  and FoxO (11, 12). VEGF-A is a well-established target of HIF-1 $\alpha$  (21). Inhibition of Mdm2 activity by Nutlin-3a or Mdm2-knockdown can reduce HIF-1 $\alpha$  stabilization and VEGF-A gene expression (9,24). Phosphorylation of Mdm2 at Ser166 has been demonstrated to enhance HIF-1 $\alpha$  stability and activation under hypoxic conditions (25). Similarly, a phosphomimetic form of Mdm2 promotes HIF-1 $\alpha$ -dependent transcription of VEGF-A in response to growth factor stimulation (26).

A few recent studies have suggested that FoxO proteins down-regulate VEGF-A mRNA (19, 20). The effect of the phosphomimetic Mdm2 on VEGF-A expression is prevented by overexpression of FoxO1 (26). Our group reported that Mdm2 phosphorylation on Ser166 in ECs stimulated with growth factors or shear stress increases Mdm2-FoxO1 interaction and reduces FoxO1 protein levels (12). We also have observed that overexpression of FoxO1 causes a reduction in VEGF-A mRNA (20). In the current study, we measured no change in the binding between Mdm2 and p53 or HIF-1 $\alpha$  when comparing human primary microvascular ECs overexpressing WT-Mdm2 or S166D-Mdm2. However, more Mdm2 protein was pulled down with a FoxO1 antibody in S166D-Mdm2 cells than in WT-Mdm2 cells, suggesting an increased interaction

between these 2 proteins. Thus, our data suggest that FoxO1 is a more relevant target of Mdm2 in non-hypoxic ECs.

Although other studies have supported the idea that Mdm2 is an upstream regulator of VEGF-A, in our present work VEGF-A-dependent phosphorylation of Mdm2 at Ser166 was an important mediator of a broad pattern of proangiogenic signaling within ECs. We have shown clear evidence that VEGF-A-induced phosphorylation of Mdm2 on Ser166 is associated with increased binding to FoxO1 and a reduced transcription of the FoxO target genes Sprouty-2, p27, and TSP-1 in primary ECs (13, 18). Sprouty-2 is an inhibitor of MAPK signaling, whereas p27 mediates cell cycle arrest (18). TSP-1 is a potent antiangiogenic factor associated with inhibition of EC migration and proliferation (13). The expression levels of FoxO1 targets TSP-1, p27, and Sprouty-2 all were decreased in S166D-Mdm2 cells. We observed that ECs overexpressing S166D-Mdm2 exhibited a down-regulation of VEGF-A mRNA compared with WT-Mdm2 cells, which was counter to the expected effect. Because Mdm2 activity has been shown to both down-regulate FoxO and stabilize HIF-1 $\alpha$ , we hypothesized that constitutive activation of Mdm2 would result in enhanced VEGF-A expression. However, Mdm2 also exerts posttranscriptional control of VEGF-A by interacting with the 3'-UTR region of VEGF-A mRNA in the cytoplasm, which increases transcript stability and leads to increased protein production. However, this influence is enhanced by expression of the nonphosphorylated form of Mdm2, which remains cytoplasmic (27). Thus, prolonged overexpression of S166D-Mdm2, which localizes to the nucleus, could have the effect of reducing VEGF-A mRNA stability, which underlies the significant reduction of VEGF-A mRNA observed in these cells when compared to WT-Mdm2 cells. Further investigation would be needed to determine whether this mechanism occurs in response to exercise-induced phosphorylation. Overall, stimulation of primary human microvascular ECs with recombinant

VEGF-A or expression of S166D-Mdm2 increased the angioadaptive VEGF-A:TSP-1 ratio, suggesting the promotion of a proangiogenic phenotype in these cells.

We examined the role of Ser166 phosphorylation on cell migratory activity because it is a key step of the angiogenic process. Past work indicated that the major effect of Nutlin-3a on ECs was inhibition of cell migration rather than cell proliferation or cell survival (28). In the current study, we used a 3D ex vivo muscle explant model to demonstrate that exercise-driven skeletal muscle EC migration was impaired by Nutlin-3a. This finding suggests that Mdm2 activity is necessary for exercise-induced endothelial outgrowth and was confirmed in vitro by using human primary microvascular ECs. Nutlin-3a treatment fully abolished the stimulatory effect of VEGF-A, but only partially abolished the response induced by serum and ECGS. This result leads to the proposal that Mdm2 is a critical downstream effector of VEGF-A in human primary ECs. Migratory activity was enhanced in cells transduced with S166D-Mdm2, even in the absence of a growth factor stimulus, indicating a specific role of Ser166 phosphorylation in the modulation of EC migration. We have provided evidence that stimulation of ECs with VEGF-A promotes numerous proangiogenic alterations in gene expression that could influence cell migration. Phosphorylation of Mdm2 at Ser166 also could initiate rapid changes in migratory phenotype by directly influencing cell migratory signal pathways, but to date, potential mechanisms through which this might occur remain to be established.

In conclusion, our findings represent a major advance in our understanding of the physiologic regulation of Mdm2 activity during exercise by identifying VEGF-A as an upstream regulator of Ser166 phosphorylation. Our work revealed a novel exercise-induced VEGF-A-Mdm2-FoxO pathway that has the potential to control the angiogenic process in skeletal muscle. This finding revisits the previously established relationship between Mdm2 and VEGFA,

suggesting that these 2 molecules interact with each other in a complex and reciprocal manner in the physiologic milieu. This study provides a foundation for future research to explore potential clinical and therapeutic advantages of modulating Mdm2 angiogenic function in chronic diseases where skeletal muscle capillarity limits exercise capacity and affects disease prognosis.

### **Authors Contribution**

This work was supported by Grant RGPIN341258 from the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research. G.D. received a scholarship from Fonds de Recherche du Quebec–Sante (FRQS). G.G. is recipient of an Investigator Award from FRQS. E.R., J.A., and O.B. formulated and designed the experiments. J.A., E.R., J.C., G.D., J.V., A.S., and O.B. performed the experiments. E.R., J.A., and O.B. analyzed the data. T.H., G.G., T.G., I.M.O., and O.B. contributed reagents, materials, and analytical tools. J.A., E.R., and O.B. wrote the manuscript. All authors contributed significantly to the editing. The authors declare no conflicts of interest.

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## Figure legends

Figure 7.1 Physical exercise stimulates VEGF-A expression and Mdm2 phosphorylation in skeletal muscle. A–D) Immunoblots (A,C) and densitometry analyses (B, D) for the p-Ser166-Mdm2, Mdm2, and VEGF-A in vastus lateralis muscle biopsy specimens from healthy human subjects (A, B; n = 5 subjects) and in plantaris muscles from Sprague-Dawley rats (C, D; n = 12 animals per group).  $\beta$ -Actin was used as a loading control. Human biopsy specimens were collected at rest, pre-exercise (pre), immediately after exercise (post), and 2 h after exercise. Rat muscles were collected from sedentary animals, immediately after exercise, or 4 h after exercise. Data are means  $\pm$  SEM. \* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$  vs. pre-exercise group, by 1-way ANOVA. E) Correlation between VEGF-A and p-Ser166-Mdm2 protein levels in rat plantaris muscle (n = 8 animals).

Figure 7.2 VEGF-A stimulates in vivo Mdm2 phosphorylation on Ser166 in response to 1 bout of exercise. A) Immunoblots of VEGF-A in gastrocnemius muscles from WT mice and mice deficient in VEGF-A expression in skeletal myofibers (mVEGF<sup>-/-</sup>).  $\beta$ -Actin was used as a loading control. B) The capillary: fiber ratio was determined after isolectin staining in gastrocnemius cross sections from WT and mVEGF<sup>-/-</sup> mice. Data are means  $\pm$  SEM (n = 7 WT and n = 9 mVEGF<sup>-/-</sup> mice). \*\*\* $P \leq 0.001$  WT vs. mVEGF<sup>-/-</sup> mice. C, E) Immunoblots for VEGF-A (C) and p-Ser166-Mdm2 and Mdm2 (E) in gastrocnemius muscles from sedentary (Sed.) and exercised (Ex.) WT and mVEGF<sup>-/-</sup> mice. D, F, G) Densitometry analyses for VEGF-A (D), p-Ser166-Mdm2 (F), and Mdm2 (G) from (C, E).  $\beta$ -Actin was used as a loading control. H) Ratio of p-Ser166-Mdm2 to Mdm2 quantifications obtained in (F, G). Data are means  $\pm$  SEM (n = 3–5 mice/group); 2-way ANOVA shows an overall effect of VEGF-A deletion on the protein expression of VEGF-A, #  $P \leq 0.05$ ; p-

Ser166-Mdm2, #  $P \leq 0.05$ ; and the p-Ser166-Mdm2:Mdm2 ratio, ###  $P \leq 0.001$ . \*  $P \leq 0.05$  and \*\*  $P \leq 0.01$ , sedentary vs. exercise WT mice in each genotype.

Figure 7.3 VEGF-A stimulates Mdm2 phosphorylation on Ser166 in ex vivo incubated muscle specimens. A, B) Immunoblot (A) and densitometry analysis (B) of the p-Ser166-Mdm2 in response to recombinant VEGF-A stimulation (recVEGF<sub>165</sub>; 0–50 ng/ml) (n = 4 specimens per condition).  $P \leq 0.001$ ; \* $P \leq 0.01$ ; \*\* $P \leq 0.001$ ; \*\*\* $P \leq 0.0001$  vs. no rVEGF-A;  $P \leq 0.01$  vs. 50 ng/ml VEGF-A, by 1-way ANOVA. C, D) Immunoblot (C) and densitometry analysis (D) of p-Ser166-Mdm2 after stimulation with 10% sedentary or exercised muscle homogenate in the presence of KRN633 inhibitor (200 nM)  $\beta$ -Actin was used as a loading control. Results are means  $\pm$  SEM (n = 6 specimens per condition). \* $P \leq 0.05$  vs. sedentary homogenate stimulation, by 2-way ANOVA.

Figure 7.4 VEGF-A stimulates Mdm2 phosphorylation on Ser166 in primary microvascular ECs. A) Immunoblot and densitometry analysis of p-Ser166-Mdm2 in rat SMECs stimulated in vitro with increasing doses of recVEGF<sub>165</sub> (0–100 ng/ml). Means  $\pm$  SEM are represented.  $\beta$ -Actin was used as a loading control. B) Immunoblots and densitometry analyses of p-Ser166-Mdm2 and Mdm2 in primary mouse SMECs and in human primary HAMECs and HDMECs stimulated with recVEGF<sub>165</sub> (100 ng/ml).  $\beta$ -Actin was used as a loading control. Data are means  $\pm$  SEM, normalized to untreated condition within each group (n = 3 per group). \* $P \leq 0.05$  vs. untreated, by unpaired Student's t test.

Figure 7.5 Nutlin-3a inhibits exercised-induced EC outgrowth in skeletal muscle. A) Illustration of the 3D muscle explant assay. B) Representative muscle explant. The intensity of EC staining was measured between the red line (edge of the muscle biopsy) and the yellow line (the edge of the migration front) represented by (b); (a) indicates the muscle biopsy (not included in the

quantification). The intensity of fluorescence estimates the quantity of migrated ECs. C) Representative pictures of EC outgrowth from sedentary and exercised rat plantaris specimens embedded in collagen gel in the presence or absence of Nutlin-3a (1  $\mu$ M). Endothelial cells were detected by staining with TRITC-conjugated isolectin-B4. D) Quantification of EC outgrowth. Data are means  $\pm$  SEM (2 experiments, each with n = 4 specimens treated with nutlin-3a and n = 6 untreated). P = 0.0008; 2-way ANOVA indicates an interaction between the effects of Nutlin-3a and exercise stimulation. \*\*\*P  $\leq$  0.001 vs. specimens from sedentary rats without Nutlin-3a, by Newman-Keuls multiple-comparisons test.

Figure 7.6 Nutlin-3a inhibits VEGF-A-induced EC migration in vitro. A) Representative immunoblots and densitometry analyses of protein levels for Mdm2, p-Ser166-Mdm2, and  $\beta$ -actin in HDMECs in response to stimulation with recVEGF<sub>165</sub> (100 ng/ml).  $\beta$ -Actin was used as a loading control. Data are means  $\pm$  SEM, normalized to untreated group (n = 3 for untreated and n = 4 for recVEGF<sub>165</sub> treated). \*\*P  $\leq$  0.01 vs. untreated. B) Representative pictures and data analysis of the Boyden chamber migration assay with HDMECs in response to recVEGF<sub>165</sub> (100 ng/ml) stimulation, with or without the Mdm2 functional inhibitor Nutlin-3a (10  $\mu$ M). Data are means  $\pm$  SEM from 3 independent experiments, each of them with 6 migration wells/condition, 4 cell counts/well. Data for each condition are normalized to the no-recVEGF<sub>165</sub>, no-Nutlin-3a condition. Both VEGF (P  $\leq$  0.0001) and Nutlin-3a treatment (P  $\leq$  0.0001) had an overall effect, by 2-way ANOVA and post hoc comparison. \*\*\*P  $\leq$  0.001 vs. all other conditions. C) Same as in (B) by replacing recVEGF<sub>165</sub> by a stimulation with 10% FBS and 5% ECGS. Both VEGF (P  $\leq$  0.0001) and Nutlin-3a treatment (P  $\leq$  0.0001) had an overall effect, 2-way ANOVA and post hoc comparison. \*\*\*P  $\leq$  0.001 vs. nonstimulation in each respective condition; †P  $\leq$  0.001, no nutlin-3a stimulation vs. nutlin-3a stimulation.

Figure 7.7 Mdm2 phosphorylation on Ser166 stimulates EC migration. A, B) Representative immunoblots (A) and densitometry analyses (B) for the protein expression level of Mdm2 in HDMECs overexpressing WT-Mdm2. Noninfected cells served as controls.  $\beta$ -Actin was used as a loading control. Data are means  $\pm$  SEM vs. noninfected (n = 5 experiments/cell line). \*\*\*P  $\leq$  0.001; # P  $\leq$  0.05 vs. S166D-Mdm2, by 1-way ANOVA. C) Representative immunoblots for the protein level of p-Ser166-Mdm2 in HDMECs overexpressing p-Ser166D-Mdm2. Noninfected cells served as controls.  $\beta$ -Actin was used as a loading control. D) Representative micrographs of cells described in (A), after migration in the Boyden chamber assay in response to stimulation with 10% FBS + 5% ECGS. E) Quantification of the migration shown in (B). Data are means  $\pm$  SEM from 2 independent experiments (n = 4–6 wells per condition). Noninfected, WT-Mdm2, and S166D-Mdm2; 4 cell counts/well. One-way ANOVA, significantly different from the no-stimulation condition in each cell type. \*\*\* P  $\leq$  0.001, vs. other cell types under similar stimulation conditions. # P  $\leq$  0.05, ### P  $\leq$  0.001, 1% FBS vs. 10% FBS / 5% ECGS.

Figure 7.8 Phosphorylation on Ser166-enhanced Mdm2 binding to FoxO1 leading to the down-regulation of FoxO target genes. A) After immunoprecipitation (IP) of FoxO1 in WT-Mdm2 and S166D-Mdm2 HDMECs treated with MG132, levels of FoxO1 and Mdm2 were measured by immunoblot analysis in whole-cell lysate (input) and in the IP products. Normal rabbit IgG was used as a control. The Mdm2;FoxO1 ratio expressed in raw values and as fold change in Mdm2 protein intensity between WT-Mdm2 and S166D-Mdm2 HDMECs is indicated. Data are means  $\pm$  SEM (n = 2 per group). \* P  $\leq$  0.05, WT-Mdm2 vs. S166D-Mdm2, by unpaired Student's t test. B–E) mRNA levels for p27 (B), TSP-1 (C), sprouty-2 (D), and VEGF-A (E) in WT-Mdm2 and S166DMdm2 cells, relative to the HPRT housekeeping gene. F) VEGF-A:TSP-1 ratio mRNA in

WT-Mdm2 and S166D-Mdm2 cells. B–F) Data are means  $\pm$  SEM (n = 6 per group). \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$  WT-Mdm2 vs. S166D-Mdm2, by unpaired Student's t test.

Figure 7.9 VEGF-A stimulation affects FoxO target genes and the VEGF-A:TSP-1 ratio in primary human ECs. HDMECs were stimulated with recVEGF<sub>165</sub> (100 ng/ml) for 1, 3, and 6 h. mRNA levels were measured for p27 (A), TSP-1 (B), VEGF-A (C), and VEGF-A:TSP-1 ratio (D) relative to HPRT housekeeping gene expression. Data are means  $\pm$  SEM (n = 3). One-way ANOVA shows an overall effect of VEGF-A stimulation on the mRNA expression of p27 ( $P = 0.0039$ ), TSP-1 ( $P = 0.0099$ ), VEGF-A ( $P = 0.0109$ ), and VEGF-A/TSP-1 ratio ( $P = 0.0063$ ). \*  $P \leq 0.05$  and \*\*  $P \leq 0.01$  vs. nonstimulated (0).

Figure 7.1

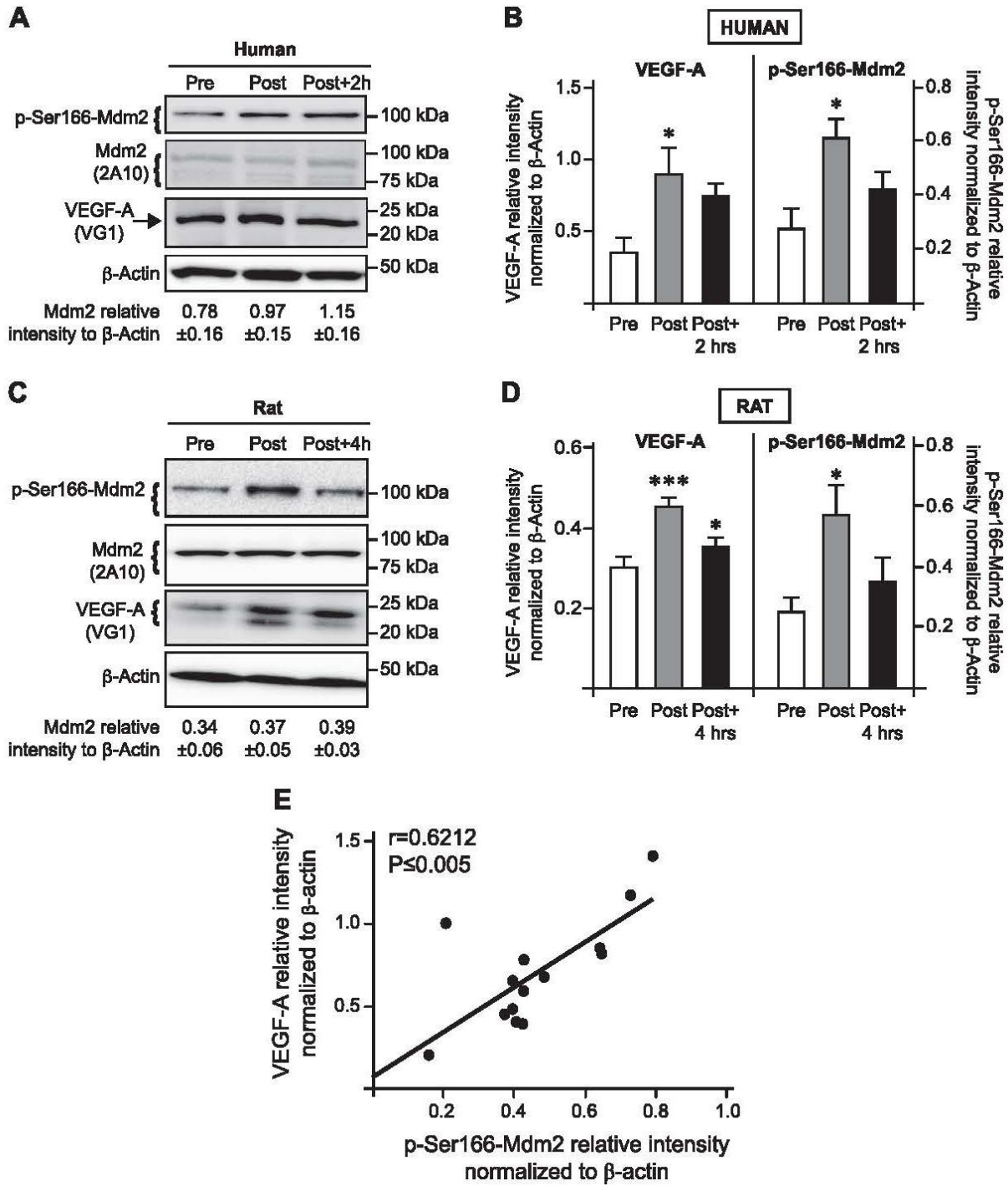


Figure 7.2

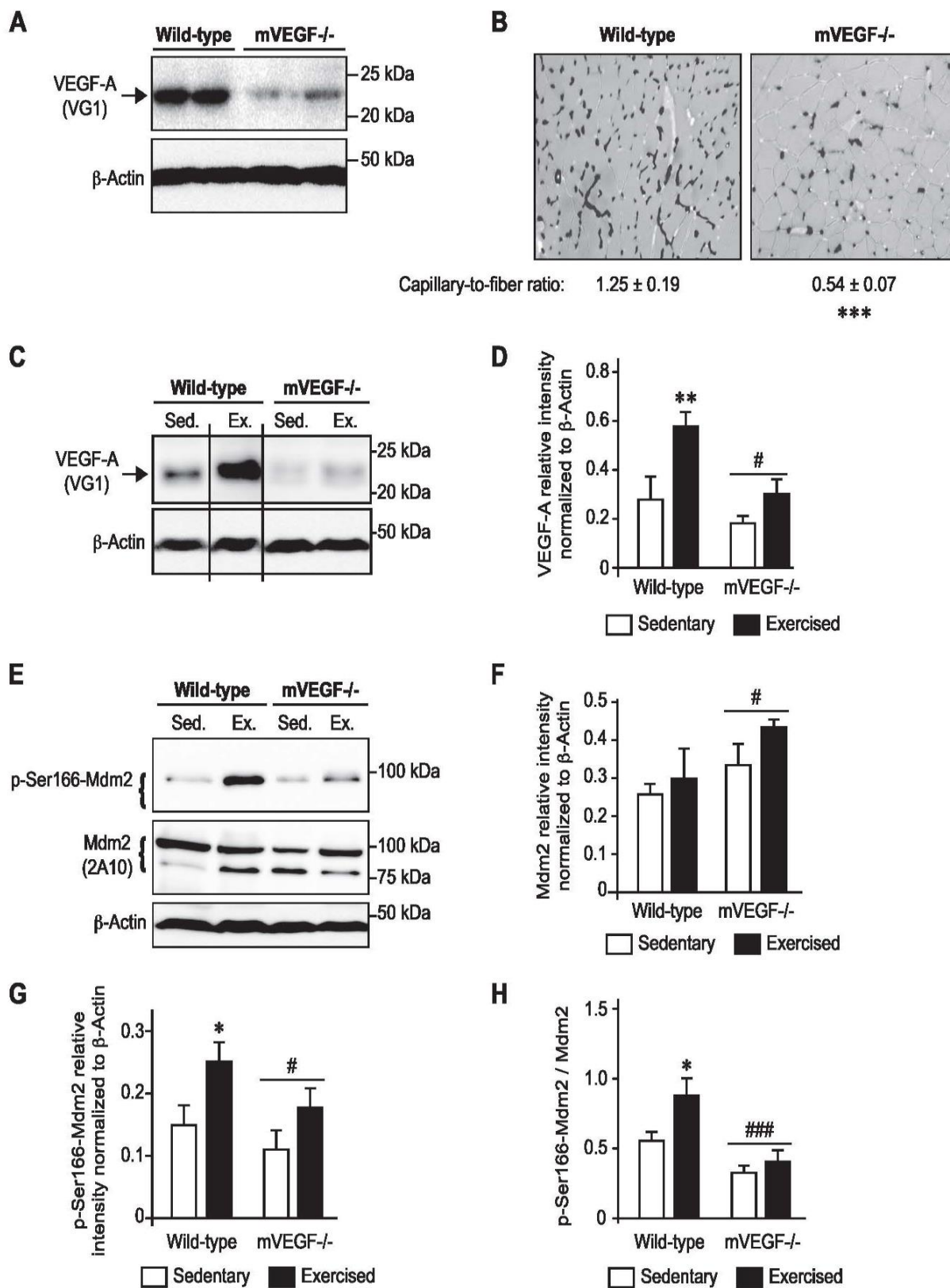
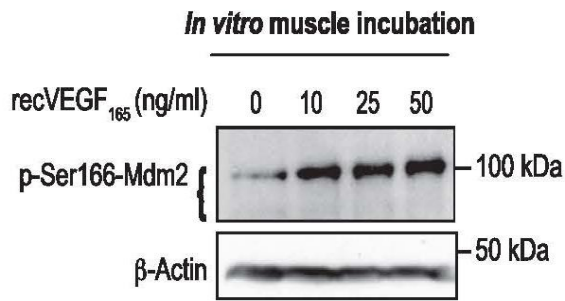


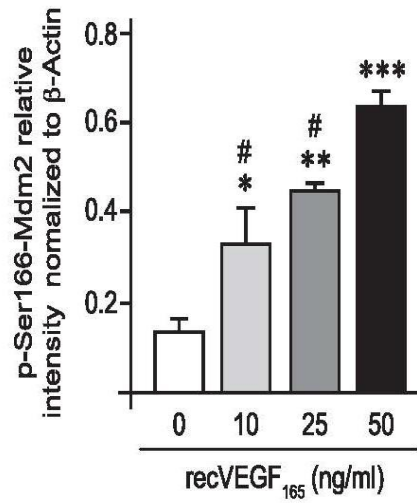


Figure 7.3

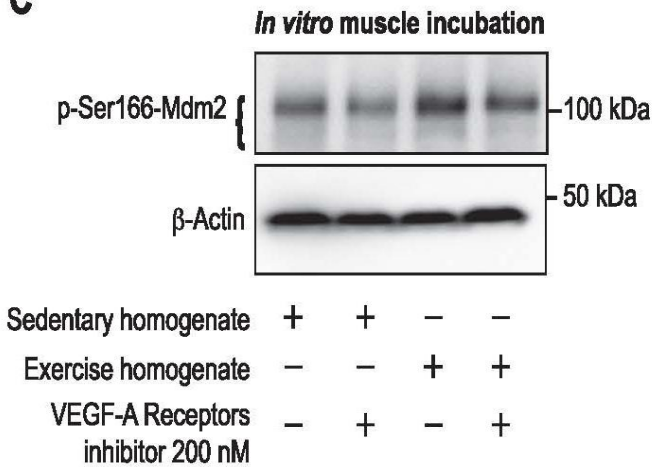
**A**



**B**



**C**



**D**

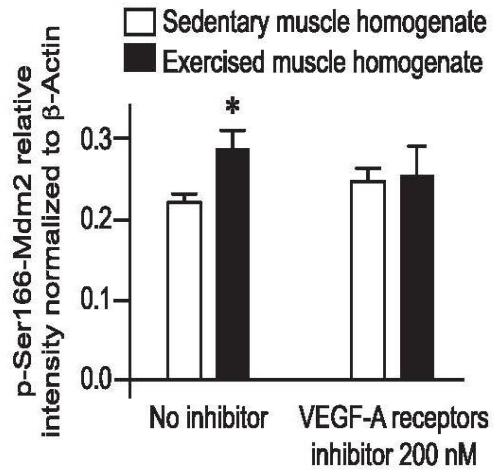


Figure 7.4

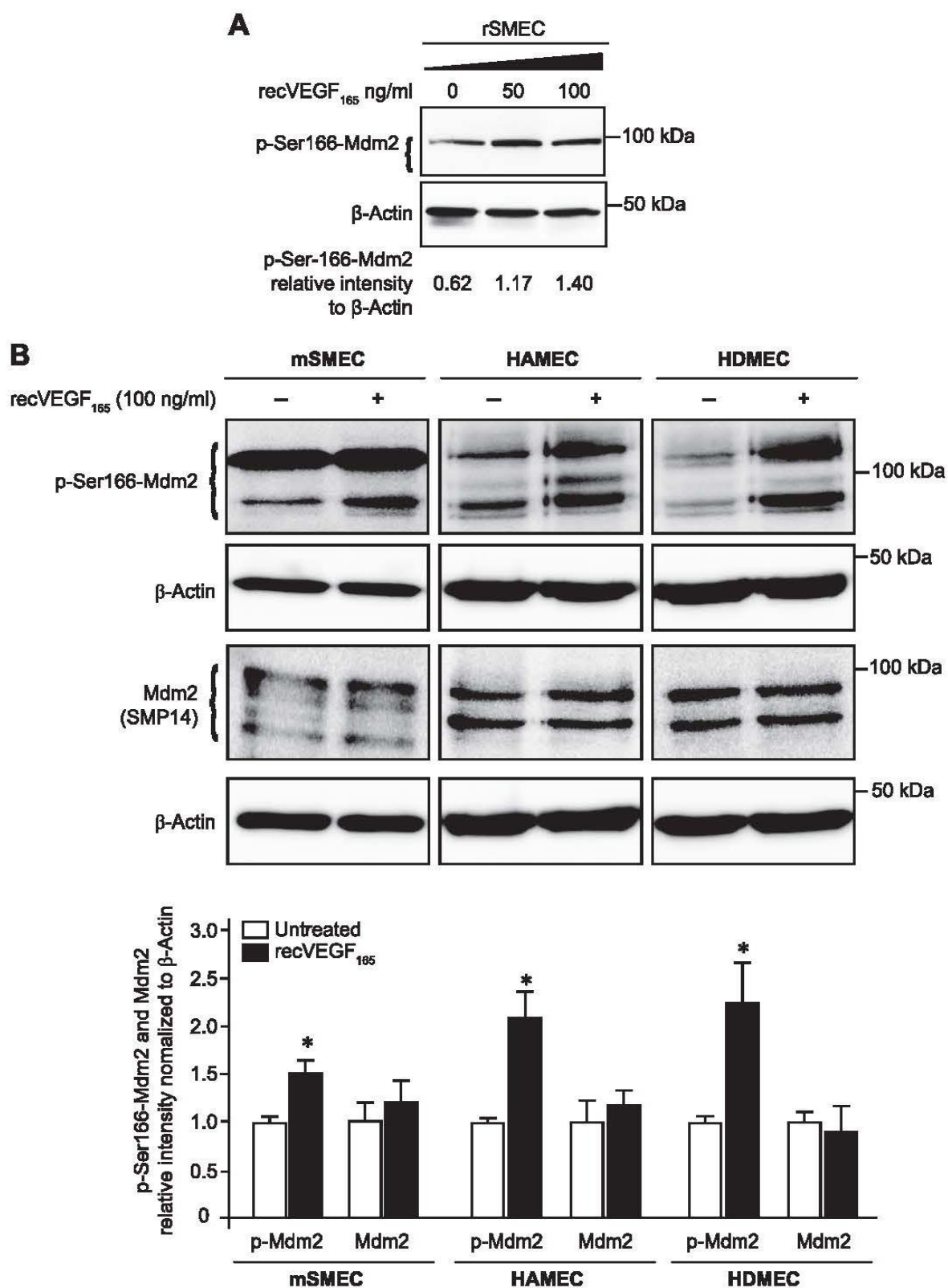


Figure 7.5

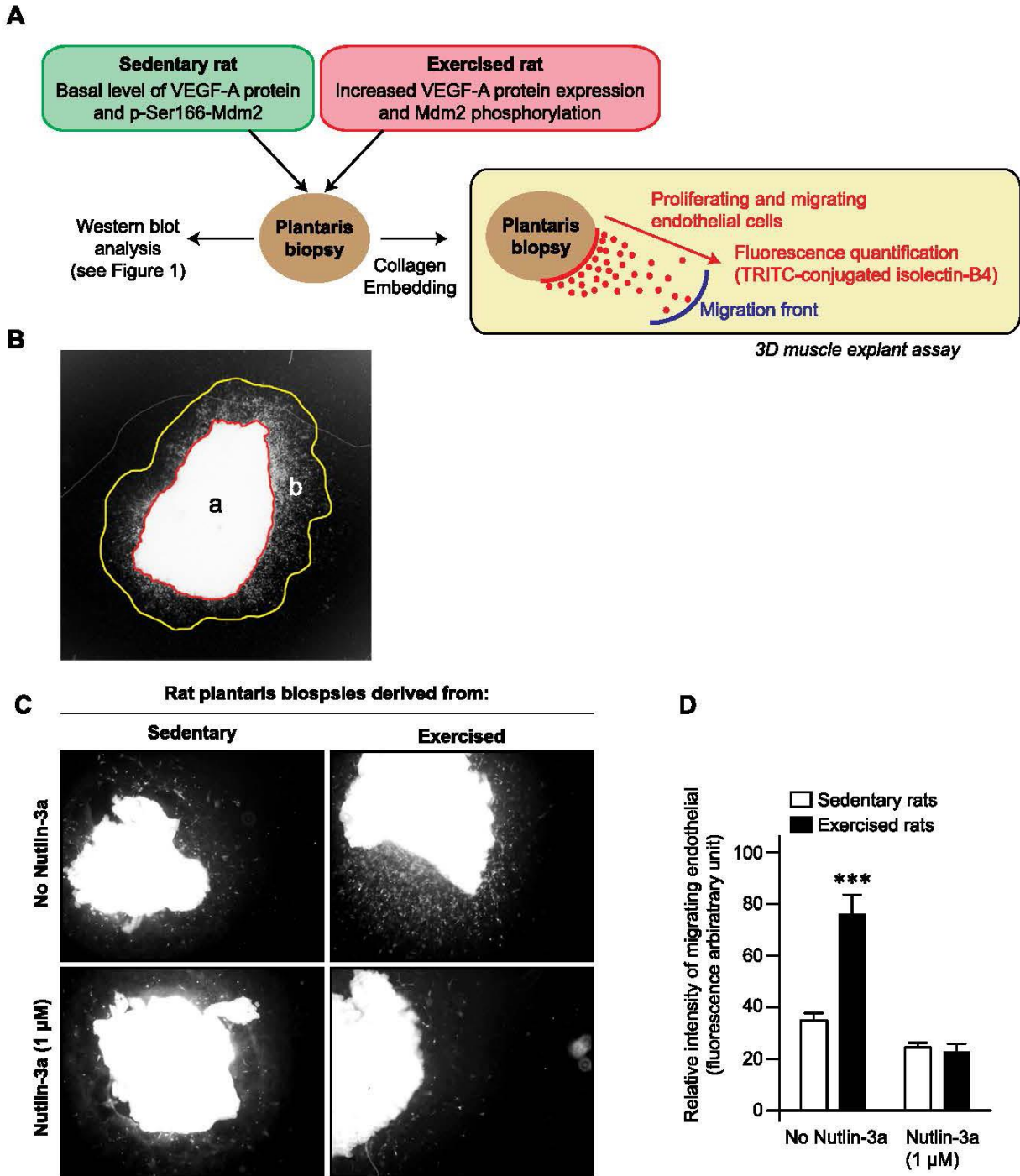


Figure 7.6

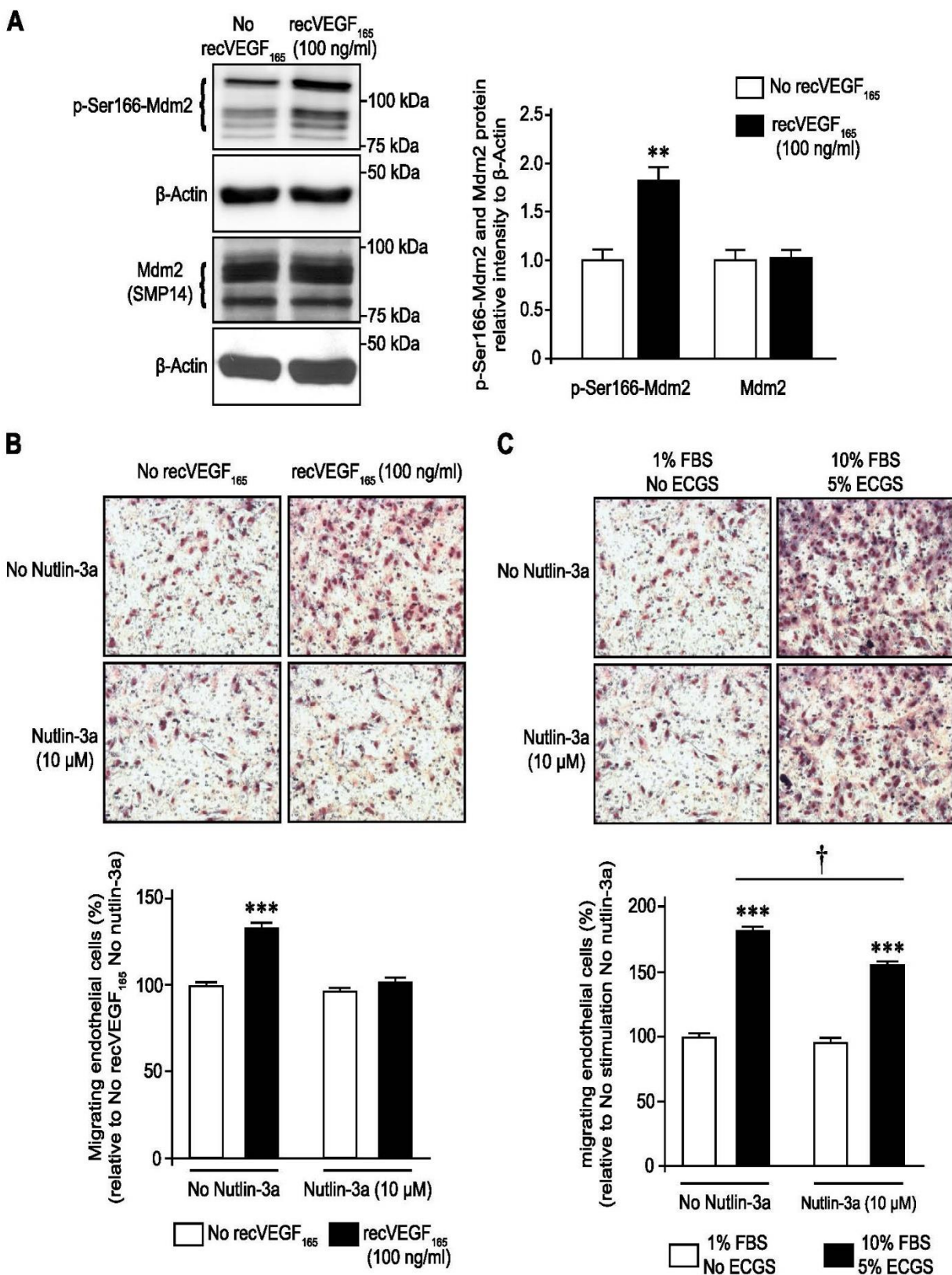




Figure 7.7

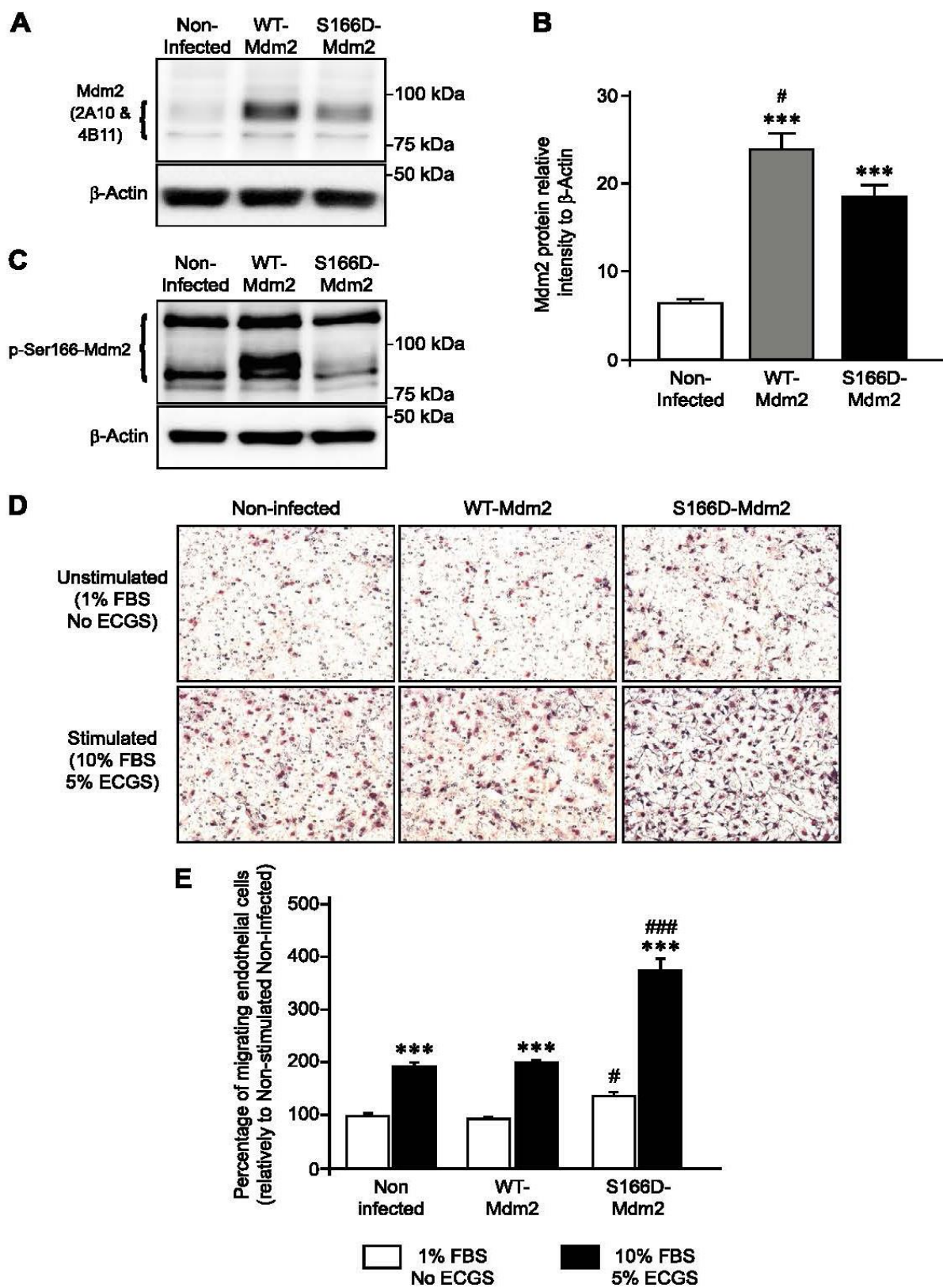


Figure 7.8

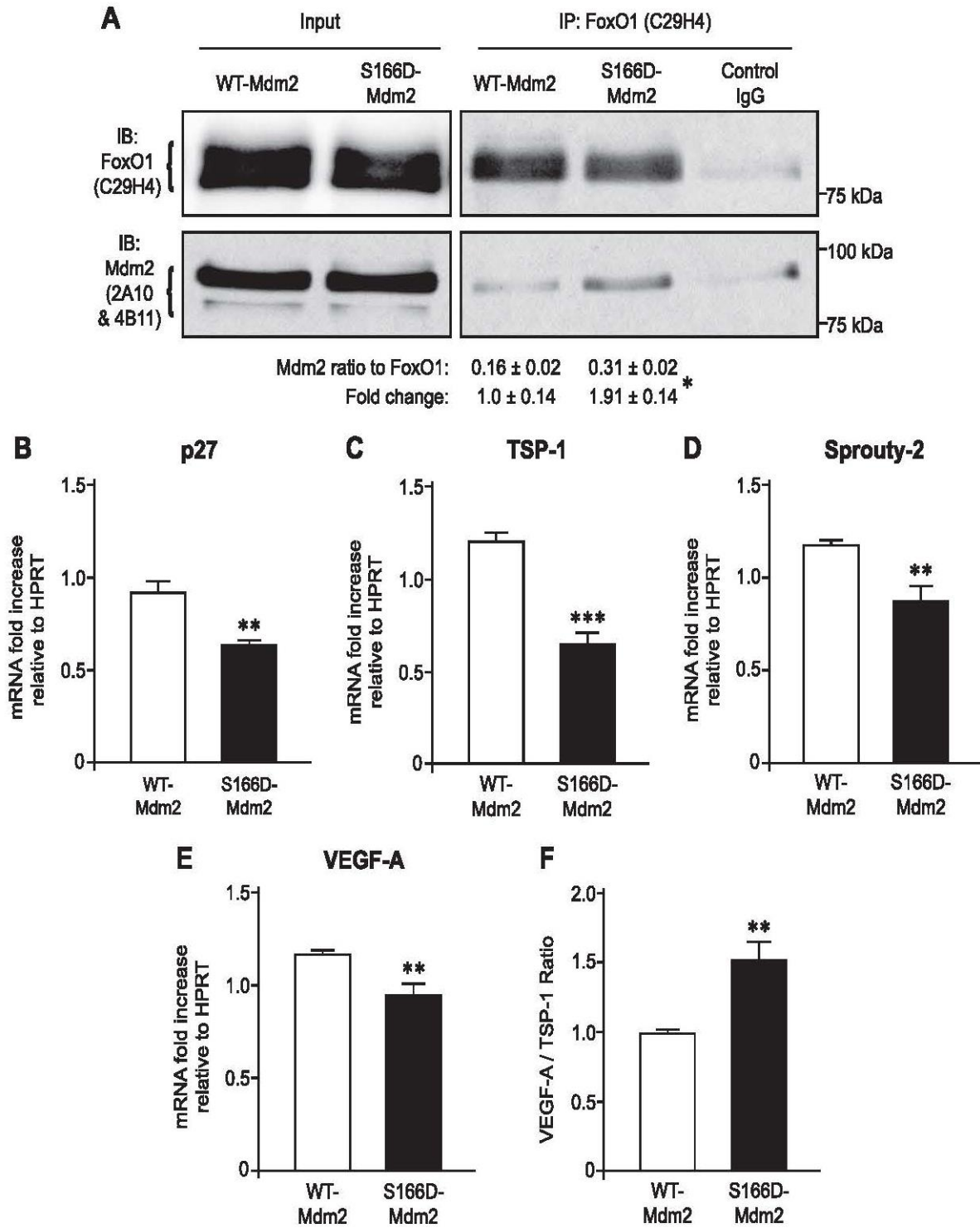
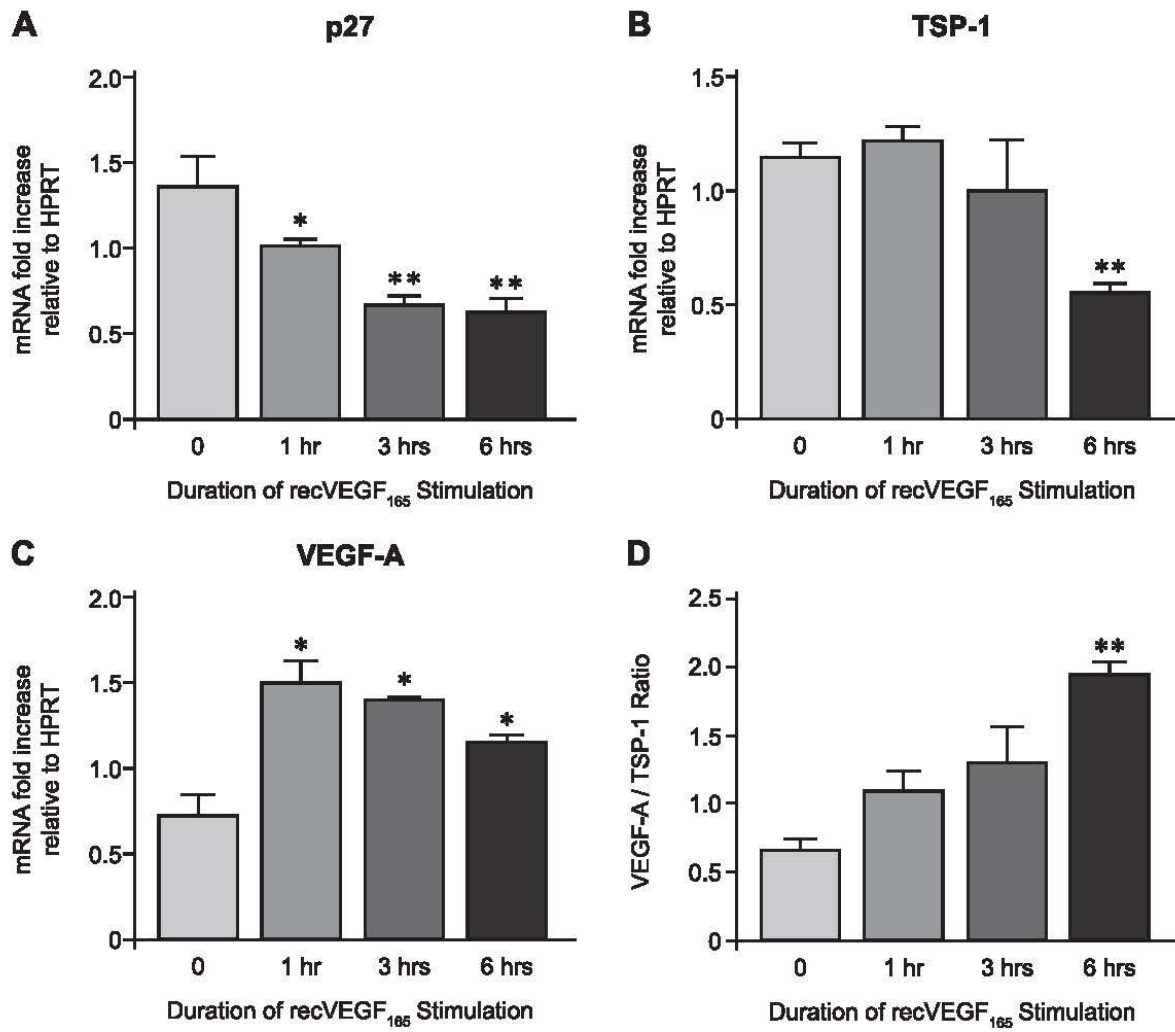
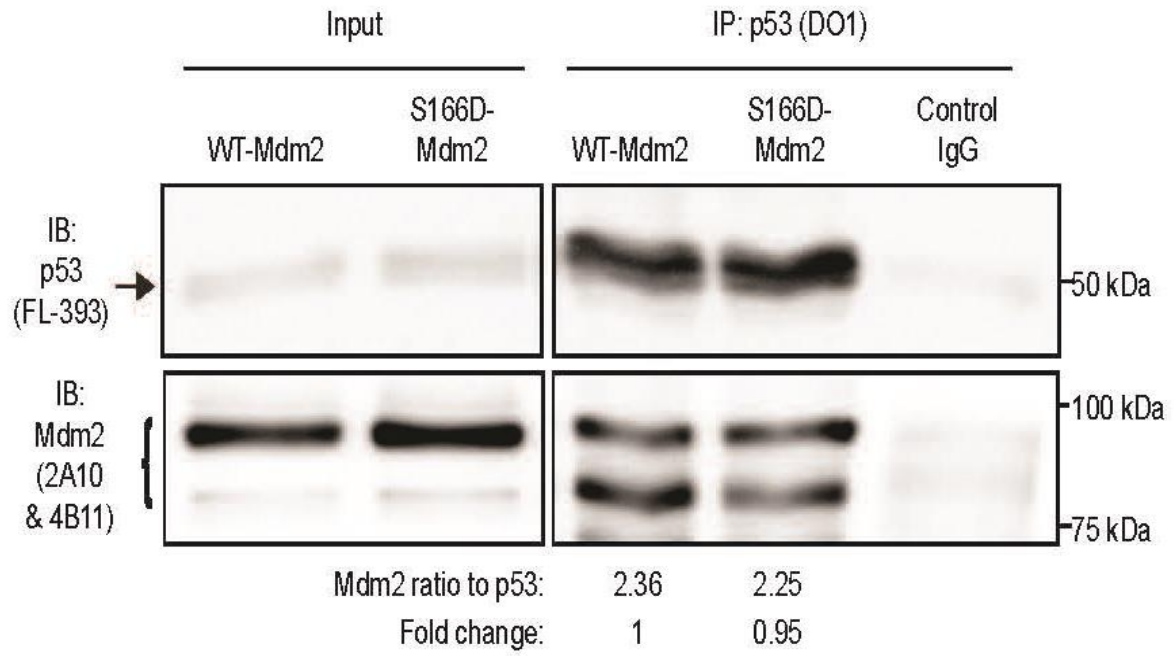


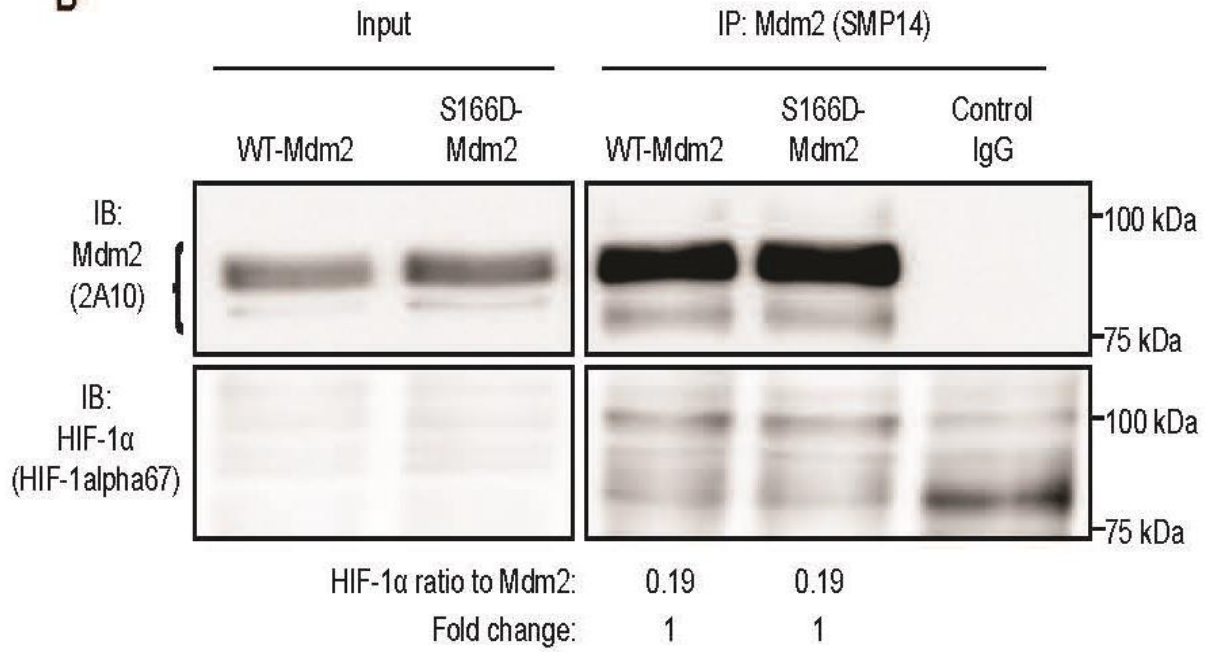
Figure 7.9



**A**



**B**





## **8. ACADEMIC RESEARCH PAPER 3**

### **The Vascular Endothelial Growth Factor-A phosphorylates Murine Double Minute-2 on its Serine 166 via the Extracellular Signal-Regulated Kinase 1/2 and p90 Ribosomal S6 Kinase in primary human endothelial cells**

**Julian Aiken** & Olivier Birot

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#### **Author contributions**

Julian Aiken:

- Contributed to formulating and designing experiments
- Performed all experiments
- Contributed to all data analysis
- Performed all statistical analysis
- Contributed in the preparation of the manuscript and all figures

#### **Scientific context:**

- We have identified VEGF-A as an important stimulator of Mdm2 phosphorylation on its serine 166
- This activation of Mdm2 enhances endothelial cell migration and increased interaction between Mdm2 and FoxO1, resulting in the down-regulation of TSP-1
- It is unclear which molecular signaling pathway(s) could be regulating VEGF-A-driven Mdm2 phosphorylation

- On the surface of endothelial cells, VEGF-A acts on its receptor VEGF-A receptor 2 (VEGFR2) [202], initiating a series of intracellular signaling cascades leading to several pro-survival and pro-angiogenic outcomes [203]
- Conveying this VEGF-A-dependent signaling are intracellular kinases that have been shown to be activated (i.e. phosphorylated) by both VEGF-A and exercise
- Previous research suggests that Akt, extracellular signal related kinases 1/2 (ERK1/2) and p90 ribosomal s6 kinase (p90RSK) can directly or indirectly induce Mdm2 phosphorylation on its serine 166, and that these kinases have been shown to be responsive to VEGF-A signaling and exercise

**Research aims:**

- Determine which kinase(s) are involved in VEGF-A-driven Mdm2 phosphorylation in primary human endothelial cells
- Utilize a time-course stimulation of recombinant VEGF-A (recVEGF-A) protein in primary human endothelial cells with and without kinase-specific pharmacological inhibitors

**Summary of main findings:**

- Time-course stimulation of primary human endothelial cells with recVEGF-A led to increased VEGFR2, Mdm2, Akt, ERK1/2, and p90RSK phosphorylation
- Following treatment with VEGF-A protein, inhibition of Akt phosphorylation by wortmannin treatment resulted in further Mdm2 phosphorylation
- Pharmacological inhibition of ERK1/2 and p90RSK led to significant attenuation of Mdm2 phosphorylation in response to VEGF-A stimulation

- VEGF-A-driven Mdm2 phosphorylation on its serine 166 is dependent on the ERK1/2-p90RSK signaling pathway in human endothelial cells

**The Vascular Endothelial Growth Factor-A phosphorylates Murine Double Minute-2 on its  
Serine 166 via the Extracellular Signal- Regulated Kinase 1/2 and p90 Ribosomal S6  
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## **Abstract**

Murine Double Minute-2 (Mdm2) has been identified as an essential regulator of skeletal muscle angiogenesis and the pro-angiogenic activity of endothelial cells. We have recently demonstrated that the pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A) is a potent upstream regulator of Mdm2 phosphorylation on its Serine 166 (p-Ser166-Mdm2), a protein modification leading to an increase in endothelial cell migration. Here, we investigated the kinase signaling pathways that could be responsible for mediating VEGF-A-dependent Mdm2 phosphorylation. Incubation of primary human dermal microvascular endothelial cells with recombinant VEGF-A for 15 min led to increased phosphorylation levels of VEGF-receptor-2, Mdm2, Akt, Extracellular Signal-Regulated Kinase 1/2 (ERK1/2), and p90 Ribosomal S6 Kinase (p90RSK) proteins. In addition to being linked to VEGF-A signaling, Akt, ERK1/2 and p90RSK have been shown to potentially lead to Mdm2 phosphorylation. We therefore next analyzed which of these kinases could be responsible for VEGF-A-dependent Mdm2 phosphorylation on Serine 166 by using kinase-specific pharmacological inhibitors. Inhibition of ERK1/2 phosphorylation by UO126 entirely abrogated the response of p-Ser166-Mdm2 to VEGF-A treatment, while Akt phosphorylation inhibition by wortmannin led to further elevations in p-Ser166-Mdm2. p90RSK has been identified as a potential candidate downstream of ERK1/2 that could induce Mdm2 Ser166 phosphorylation. Two independent p90RSK inhibitors, FMK and BI-D1870, each led to an entire loss of p-Ser166-Mdm2 responsiveness to VEGF-A. Taken together, our results demonstrate that VEGF-A driven Mdm2 phosphorylation on Ser166 is dependent on the ERK1/2/p90RSK signaling pathway in primary human endothelial cells, furthering our understanding of the complex relationship between Mdm2 and VEGF-A in a physiological context.

## Introduction

The E3 ubiquitin ligase Murine Double Minute-2 (Mdm2) is mainly known for its role as a negative regulator of the tumor suppressor p53. Interestingly, recent studies have shown that Mdm2 could also be considered as a crucial regulator of angiogenesis, i.e. the growth of blood capillaries, under pathological (cancer) [1-3] or physiological (exercise) [4-6] situations. We have recently demonstrated in vivo that Mdm2 expression level in rodent skeletal muscle was indeed indispensable both for the maintenance of established capillaries as well as for exercise-induced angiogenesis [4]. The level of Mdm2 activity has also significant implications within the endothelial cell, regulating cell proliferation [7], migration [6,7] and tube formation [7], three major cell activities required during the angiogenic process.

The Vascular Endothelial Growth Factor-A (VEGF-A) is a potent pro-angiogenic molecule that similarly to Mdm2 has been demonstrated to be required in the skeletal muscle for capillary maintenance as well as exercise-induced angiogenesis [8]. Mdm2 has been identified as an upstream regulator of VEGF-A expression through interactions with its downstream target hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), a well-established transcription factor for VEGF-A [9-11]. We have recently revisited this Mdm2-VEGF-A relationship, providing novel evidence that in addition to Mdm2 being an upstream regulator of VEGF-A expression, VEGF-A also acts as an upstream regulator of Mdm2 phosphorylation on its Serine 166 (p-Ser166-Mdm2) [6]. This site of Mdm2 phosphorylation lies in the nuclear localization sequence of Mdm2, and promotes the translocation of Mdm2 from the cytoplasm to the nucleus where it can interact with its downstream targets [12]. We have shown that one single bout of exercise leads to a significant increase in the protein expression levels of VEGF-A and p-Ser166-Mdm2 in both rodent and human skeletal muscle, however this response was impaired in myofibre-specific VEGF-A knockout mice [6]. Using an

in vitro approach to explore this relationship specifically in endothelial cells, we demonstrated that recombinant VEGF-A (recVEGF-A) protein indeed stimulates Mdm2 phosphorylation on Ser166, a modification that enhances the migratory activity of primary human endothelial cells [6]. The importance of this phosphorylation site in stimulating endothelial cell migration was confirmed by generating an endothelial cell line expressing a phospho-mimetic form of p-Ser166-Mdm2 [6]. Interestingly, the stimulatory effect of recVEGF-A on cell migration was lost when treating the cells with the Mdm2 antagonist Nutlin-3a, indicating that Mdm2 activity is required for the pro-angiogenic activity of human endothelial cells in response to VEGF-A.

Here, we aimed to identify the kinase(s) involved in VEGF-A mediated phosphorylation of Mdm2 on Ser166 in primary human microvascular endothelial cells. We focused on the main kinases previously shown in the literature to be activated by VEGF-A signaling and exercise that have also been identified as upstream regulators of Mdm2 phosphorylation on Ser166.

## **Materials and methods**

*Cell culture.* Primary human dermal microvascular endothelial cells (HDMECs) were purchased from ScienCell Research Laboratories (cat. no. 2000; Carlsbad, CA, USA). The cells were maintained, as previously described [6], in ECM (cat. no. 1001) supplemented with 5% FBS (cat. no. 0025), 1% endothelial cell growth supplement (ECGS; cat. no. 1052), and antibiotic solution containing 100 U/ml penicillin and 100 mg/ml streptomycin (cat. no. 0503; all from ScienCell). For VEGF-A stimulation time-course experiments, HDMECs were starved overnight with ECM containing 1% FBS before stimulation with recombinant human VEGF-A (#100-20; Peprotech, Rocky Hill, NJ, USA) for the indicated lengths of time. For pharmacological kinase inhibitor experiments, cells were starved overnight then pre-treated for 1 h with wortmannin (#9951; Cell

Signaling Technology), UO126 (#9903; Cell Signaling Technology), BI-D1870 (BML-EI407-0001; Enzo Life Sciences, Farmingdale, NY, USA), or FMK (#1848; Axon MedChem, Reston, VA, USA), respectively, before stimulation with recombinant human VEGF-A for 5 min.

*Western blotting.* Immunoblotting was carried out on protein extracts from primary human dermal microvascular endothelial cells as previously described [6]. Denatured samples (20 mg/well) were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and blotted onto nitrocellulose (Whatman, BA95, Sigma-Aldrich, Oakville, Ontario, Canada) membranes. Quality of the transfer was confirmed by Ponceau S red staining. After blocking with 5% fat-free milk at room temperature for 45 min, the blots were probed overnight at 4°C with primary antibodies against the following proteins: p-Ser166-Mdm2 (#3521), Akt (#9272), pser473-Akt (#4058), ERK1/2 (#4695), p-Thr202/Tyr204-ERK1/2 (#4370), RSK1/2/3 (#9355), p-Ser380-p90RSK (#9335), p-Tyr1175-VEGFR2 (#3770) and  $\alpha/\beta$ -tubulin (#2148) were from Cell Signaling Technology (Beverly, MA, USA); Mdm2 clone SMP14 (sc-965) and  $\beta$ -actin (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After incubation with the secondary antibodies HRP-linked anti-mouse antibody (cat. no. P0260; Dako, Carpinteria, CA, USA) or HRP-linked anti-rabbit antibody (#7074; Cell Signaling) proteins were visualized with enhanced chemiluminescence (Millipore) on Imaging Station 4000 MM Pro (Carestream Health, Rochester, NY, USA) or on X-ray film (CL-XPosure Film; prod. no. 34090; Thermo Scientific, Rockford, IL, USA). Blots were analyzed with Carestream software.

*Statistical analysis.* Statistical analyses were performed with 1- and 2-way ANOVAs with Prism5 (GraphPad, San Diego, CA, USA). For 1- and 2-way ANOVAs, Newman-Keuls multiple comparison and Bonferroni post hoc tests were used, respectively.  $P \leq 0.05$  was considered to be statistically significant.



## Results and discussion

The VEGF-receptor 2 (VEGFR2) is key to initiate VEGF-A-dependent intracellular signaling pathways [13]. Tyr1175 is a major VEGF-A-dependent autophosphorylation site present on VEGFR2 that initiates a variety of cellular processes including vascular permeability, cell survival and proliferation [14]. Therefore, in order to identify the kinase(s) that could link VEGF-A to p-Ser166-Mdm2, we first ensured in our culture of primary human dermal microvascular endothelial cells (HDMECs) that time-course stimulation with human recombinant VEGF-A (recVEGF-A) led to increased VEGFR2 phosphorylation at Tyr1175 (Fig. 8.1A and B,  $0.05 \pm 0.02$  at 0 min vs.  $1.99 \pm 0.13$  at 5 min (+3968% increase);  $1.77 \pm 0.24$  at 10 min (+3514%); and  $1.45 \pm 0.03$  at 15 min (+2866%); 1-way ANOVA;  $P \leq 0.0001$ ).

As expected, recVEGF-A concomitantly stimulated Mdm2 phosphorylation at Ser166 throughout the 15 min time-course with increases of respectively +122% at 5 min, +112% at 10 min, and +135% at 15 min in VEGF-A-stimulated cells versus the untreated ones (Fig. 8.1C and D;  $0.64 \pm 0.07$  at 0 min vs.  $1.42 \pm 0.11$  at 5 min;  $1.35 \pm 0.05$  at 10 min; and  $1.50 \pm 0.05$  at 15 min; 1-way ANOVA;  $P \leq 0.0001$ ), while total Mdm2 protein levels remained unaltered (Fig. 8.1C).

Phosphatidylinositol 3-kinase (PI3K) activation is well established as part of VEGF-A signaling [15], and the PI3K downstream target Akt has been widely reported, predominantly in a cancer context, to induce Mdm2 phosphorylation on Ser166 [16-18]. Thus, we investigated whether VEGF-A treatment of HDMECs could induce Akt phosphorylation on Ser473, as it is this residue on Akt that has shown to induce Mdm2-Akt association and promote Mdm2 nuclear localization [19]. Here, VEGF-A led to a significant time-course increase in Akt phosphorylation (Fig. 8.1E and F;  $0.12 \pm 0.05$  at 0 min vs.  $0.93 \pm 0.12$  at 5 min (+708%);  $0.81 \pm 0.09$  at 10 min (+600%); and  $0.78 \pm 0.07$  at 15 min (+577%); 1-way ANOVA;  $P \leq 0.001$ ). The extracellular

signal-regulated kinase 1/2 (ERK1/2) are also known as downstream targets of the VEGF-A/VEGFR2 signaling pathway [14]. Others have suggested that ERK1/2 could play an important role in the induction of p-Ser166-Mdm2 in response to various environmental and growth factors [20]. Previous studies have shown that VEGF-A stimulates the phosphorylation of ERK1/2 at Thr202/Tyr204 [21e23], and that this site of ERK1/2 activation could play a role in Mdm2 phosphorylation on Ser166 [20]. Here, we show that ERK1/2 phosphorylation at Thr202/Tyr204 is strongly induced by VEGF-A treatment in HDMECs (Fig. 8.1G and H;  $0.62 \pm 0.07$  at 0 min vs.  $1.55 \pm 0.09$  at 5 min (+152%);  $1.49 \pm 0.02$  at 10 min (+142%); and  $1.41 \pm 0.11$  at 15 min (+128%); 1-way ANOVA;  $P \leq 0.0001$ ). While ERK1/2 is phosphorylated by VEGF-A, and has been suggested to induce p-Ser166-Mdm2, there is no available evidence for a direct interaction of ERK1/2 and Mdm2. Therefore, the p90 ribosomal S6 kinase (p90RSK) could be an intriguing candidate for mediating VEGF-A directed Mdm2 phosphorylation. p90RSK lies downstream of ERK1/2 signaling [24], has been shown to be activated by VEGF-A in cultured rat cardiac myocytes and fibroblasts [25] and has been suggested to induce p-Ser166-Mdm2 in vitro [26,27]. Several phosphorylation sites have been identified on p90RSK and among them, phosphorylation of serine 380 plays a key role in mediating p90RSK activation [24]. In the present study, we found that p90RSK phosphorylated on Ser380 is significantly elevated throughout the VEGF-A treatment period (Fig. 8.1I and J;  $0.08 \pm 0.01$  at 0 min vs.  $1.64 \pm 0.06$  at 5 min (+2084%);  $1.30 \pm 0.01$  at 10 min (+1635%); and  $0.79 \pm 0.09$  at 15 min (+950%); 1-way ANOVA;  $P \leq 0.0001$ ).

To determine which of these kinases identified to respond to VEGF-A treatment in our human endothelial cells could be involved in VEGF-A-dependent Mdm2 phosphorylation on its Ser166, we incubated HDMECs with or without recVEGF-A in the presence or absence of kinase-specific pharmacological inhibitors. As an irreversible PI3K inhibitor, wortmannin is well

established as an inhibitor of Akt phosphorylation [28]. In our study, we observed a complete loss of Akt phosphorylation in response to wortmannin treatment independently of recVEGF-A stimulation (Fig. 8.2A and B;  $1 \pm 0.09$ , untreated vs.  $6.98 \pm 0.54$ , with recVEGF-A;  $0 \pm 0$  with wortmannin; and  $0 \pm 0$  with wortmannin and recVEGF-A). Interestingly, such inhibition of Akt activity led to a further induction of p-Ser166-Mdm2, both with and without the addition of recVEGF-A (Fig. 8.2A and C;  $1 \pm 0.07$ , untreated vs.  $2.66 \pm 0.15$ , with recVEGF-A;  $1.92 \pm 0.09$ , with wortmannin; and  $3.18 \pm 0.12$ , with wortmannin and recVEGF-A). One hypothesis for such an unexpected result could be that the increased Mdm2 phosphorylation observed in response to wortmannin treatment could be a result of further ERK1/2 signaling activation due to the inhibition of a parallel Akt pathway. Although not significant, we indeed observed a 46% increase in ERK1/2 phosphorylation on Thr202/Tyr204 in wortmannin treated HDMECs independent of VEGF-A, while VEGF-A treatment led to a similar level of ERK1/2 phosphorylation with and without the presence of wortmannin ( $1 \pm 0.06$ , untreated vs.  $2.16 \pm 0.33$ , with recVEGF-A;  $1.46 \pm 0.20$ , with wortmannin; and  $2.04 \pm 0.36$ , with wortmannin and recVEGF-A). In line with our observation, Malmlof et al. [20] have shown that the PI3K inhibitor LY294002 significantly induces p-Ser166-Mdm2 while concomitantly increasing the level of ERK1/2 phosphorylation on Tyr204 in hepatic cancer cells. Next, we investigated whether ERK1/2 could be responsible for Mdm2 phosphorylation in response to recVEGF-A in HDMECs. The pharmacological inhibitor UO126 is a highly selective inhibitor of both MEK 1 and 2, therefore disrupting MEK1/2-dependent ERK1/2 phosphorylation [29]. UO126 at the concentration of 20  $\mu$ M led to a partial but significant inhibition of ERK1/2 phosphorylation on Thr202/Tyr204 in response to recVEGF-A treatment in HDMECs (Fig. 8.2D and E;  $1 \pm 0.05$ , untreated vs.  $1.98 \pm 0.22$ , with recVEGF-A;  $0.14 \pm 0.06$ , with UO126; and  $0.87 \pm 0.23$ , with UO126 and recVEGF-A). This partial inhibition of p-

Thr202/Tyr204-ERK1/2 entirely abrogated the response of p-Ser166-Mdm2 to recVEGF-A (Fig. 8.2D and F;  $1 \pm 0.04$ , untreated vs.  $2.34 \pm 0.32$ , with recVEGF-A;  $0.72 \pm 0.19$ , with UO126; and  $0.96 \pm 0.26$ , with UO126 and recVEGF-A). These results identify ERK1/2 activation as essential for VEGF-A-induced Mdm2 phosphorylation on its Ser166. As the use of a PI3K inhibitor showed a further elevation in p-Ser166-Mdm2, and that the MEK1/2 inhibitor significantly attenuated Mdm2 phosphorylation in response to recVEGF-A, we narrowed our investigation downstream of ERK1/2 signaling. p90RSK represents a strong candidate for this ERK1/2 effect on Mdm2. p90RSK is phosphorylated by ERK1/2 at its C-terminal kinase domain, thus activating the N-terminal kinase domain and allowing p90RSK to subsequently phosphorylate its downstream targets [30]. We therefore hypothesized that p90RSK is the main direct kinase involved in the VEGF-A/ERK1/2 signaling pathway responsible for Mdm2 phosphorylation on Ser166. In order to provide robust evidence of the role of p90RSK in phosphorylating Mdm2 in response to VEGF-A, we used two separate pharmacological inhibitors that bind to mutually exclusive domains on the p90RSK protein. FMK has been shown to be an irreversible p90RSK inhibitor that binds to the C-terminal kinase domain of the protein [30]. As previously demonstrated, p-Ser166-Mdm2 is elevated following recVEGF-A stimulation, however this effect is entirely lost in the presence of FMK (Fig. 8.3A and B;  $1 \pm 0.05$ , untreated vs.  $1.64 \pm 0.09$ , with recVEGF-A;  $0.65 \pm 0.13$ , with FMK; and  $0.64 \pm 0.12$ , with FMK and recVEGF-A). Conversely to FMK, BID1870 is a p90RSK inhibitor that instead binds to the N-terminal kinase domain of p90RSK [30]. As with FMK treatment, BI-D1870 was found to prevent VEGF-A-driven Mdm2 phosphorylation in the HDMECs (Fig. 8.3C and D;  $1 \pm 0.11$ , untreated vs.  $2.3 \pm 0.41$ , with recVEGF-A;  $0.99 \pm 0.16$ , with BI-D1870; and  $0.84 \pm 0.13$ , with BID1870 and recVEGF-A).

It is well documented that VEGF-A mRNA and protein expression are increased in response to one single and intense bout of exercise in human skeletal muscle [31] and that interstitial VEGF-A protein levels remain significantly elevated during and following exercise [32]. Alongside the well-described increase in VEGF-A, we have recently shown that a similar exercise regimen also strongly increased p-Ser166-Mdm2 protein levels in both rodent and human skeletal muscle tissue, and that increases in these two proteins were significantly and positively correlated [6]. Furthermore, numerous studies have shown increases in ERK1/2 and p90RSK phosphorylation in rodent and human skeletal muscle tissue in response to a variety of acute resistance and endurance exercise protocols [33-37]. Although these findings were obtained at the whole muscle level, VEGF-A is a potent myokine that when released from the muscle fibre in response to an acute bout of exercise can interact with VEGFR2 present on endothelial cells. As our current findings strongly suggest, this initiation of VEGF-A signaling in the endothelial cell can lead to the phosphorylation of ERK1/2 and p90RSK, subsequently triggering an elevation in p-Ser166-Mdm2 levels. These results identify an intracellular signaling pathway that is activated by VEGF-A signaling and regulates Mdm2 phosphorylation in primary human endothelial cells. Taken together with previous work from our laboratory that demonstrated VEGF-A as a potent upstream regulator of Mdm2 phosphorylation in endothelial cells [6], these findings unveil an in-depth understanding of the intracellular mechanisms regulating the complex pro-angiogenic VEGF-A/Mdm2 relationship.

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## Figure legends

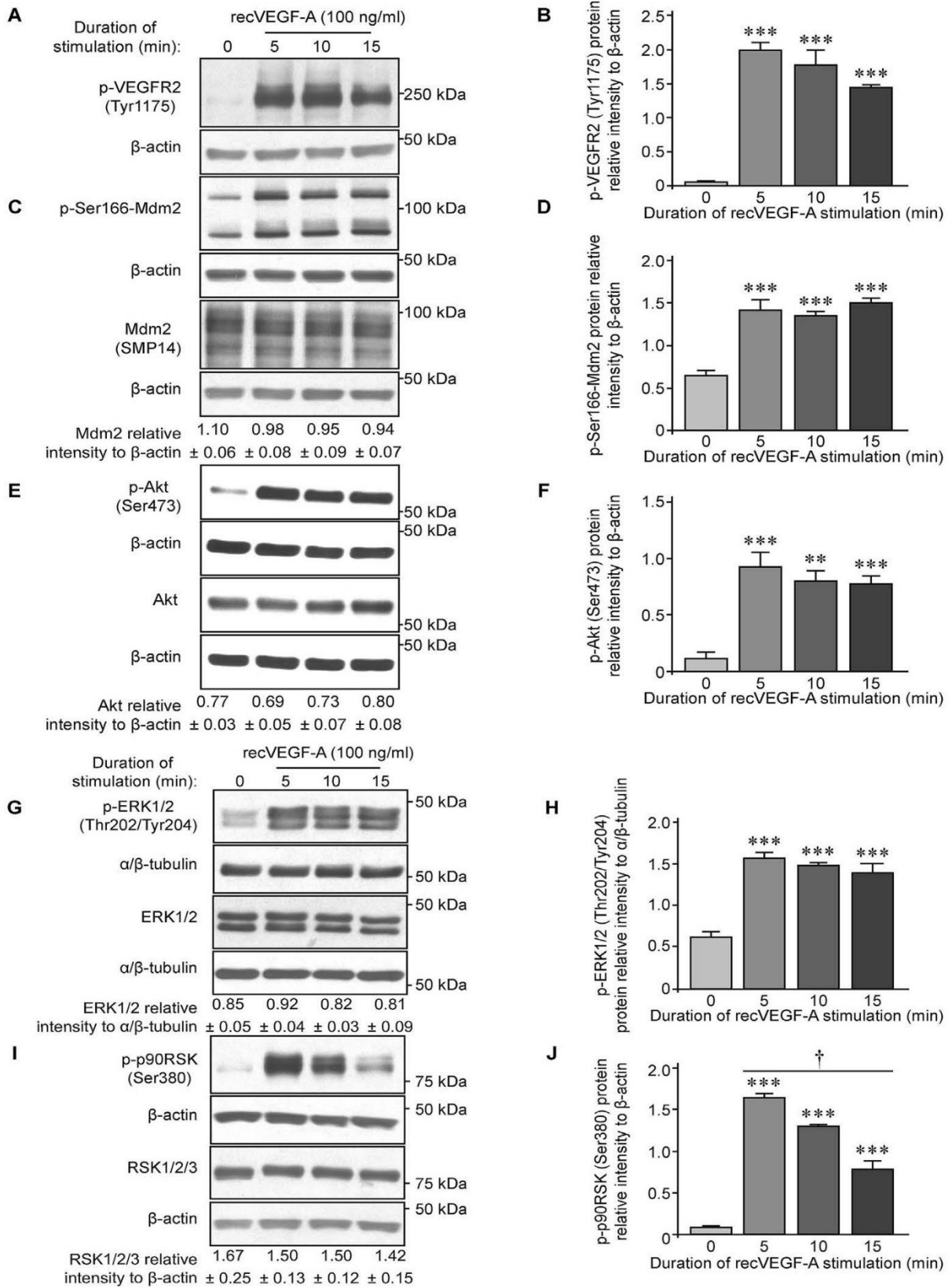
Figure 8.1 Recombinant VEGF-A treatment stimulates phosphorylation of VEGF-receptor-2, Mdm2, Akt, ERK, and p90RSK proteins in primary human endothelial cells. Immunoblotting and densitometry analysis were performed on cell lysates of primary human dermal microvascular endothelial cells (HDMECs) collected after 0 (untreated), 5, 10 or 15 min of incubation with recombinant human VEGF-A (recVEGF-A) (100 ng/ml). (A,B) Representative immunoblots and densitometry analysis for p-VEGFR2 (Tyr1175). (C,D) Representative immunoblots and densitometry analysis for p-Ser166-Mdm2 and Mdm2 protein expression. (E,F) Representative immunoblots and densitometry analysis for p-Akt (Ser473) and Akt protein expression. (G,H) Representative immunoblots and densitometry analysis for p-ERK1/2 (Thr202/Tyr204) and ERK1/2. (I,J) Representative immunoblots and densitometry analysis for p-p90RSK (Ser380) and RSK1/2/3 protein expression. In A-J,  $\beta$ -actin or  $\alpha$ / $\beta$ -tubulin were used as loading controls. Data are means  $\pm$  SEM (n = 3 samples per condition). 1-way ANOVA and Newman-Keuls multiple comparison post hoc test indicate \*\*\*  $P \leq 0.001$  vs. 0 min; †  $P \leq 0.01$  for 5 min vs. 10 min, 5 min vs. 15 min, 10 min vs. 15 min.

Figure 8.2 The MEK1/2 inhibitor UO126 attenuates VEGF-A-induced Mdm2 phosphorylation on Ser166 in primary human endothelial cells. Immunoblotting and densitometry analysis were performed on cell lysates of HDMECs collected after treatment with or without recVEGF-A (100 ng/ml) in the presence or absence of the PI3K inhibitor wortmannin (1  $\mu$ M) or the MEK1/2 inhibitor UO126 (20  $\mu$ M), respectively. (A-C) Representative immunoblots and densitometry analysis for p-Akt (Ser473), Akt, p-Ser166-Mdm2 and Mdm2 in response to recVEGF-A and wortmannin treatment. (D-F) Representative immunoblots and densitometry analysis for p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-Ser166-Mdm2 and Mdm2 in response to recVEGF-A and

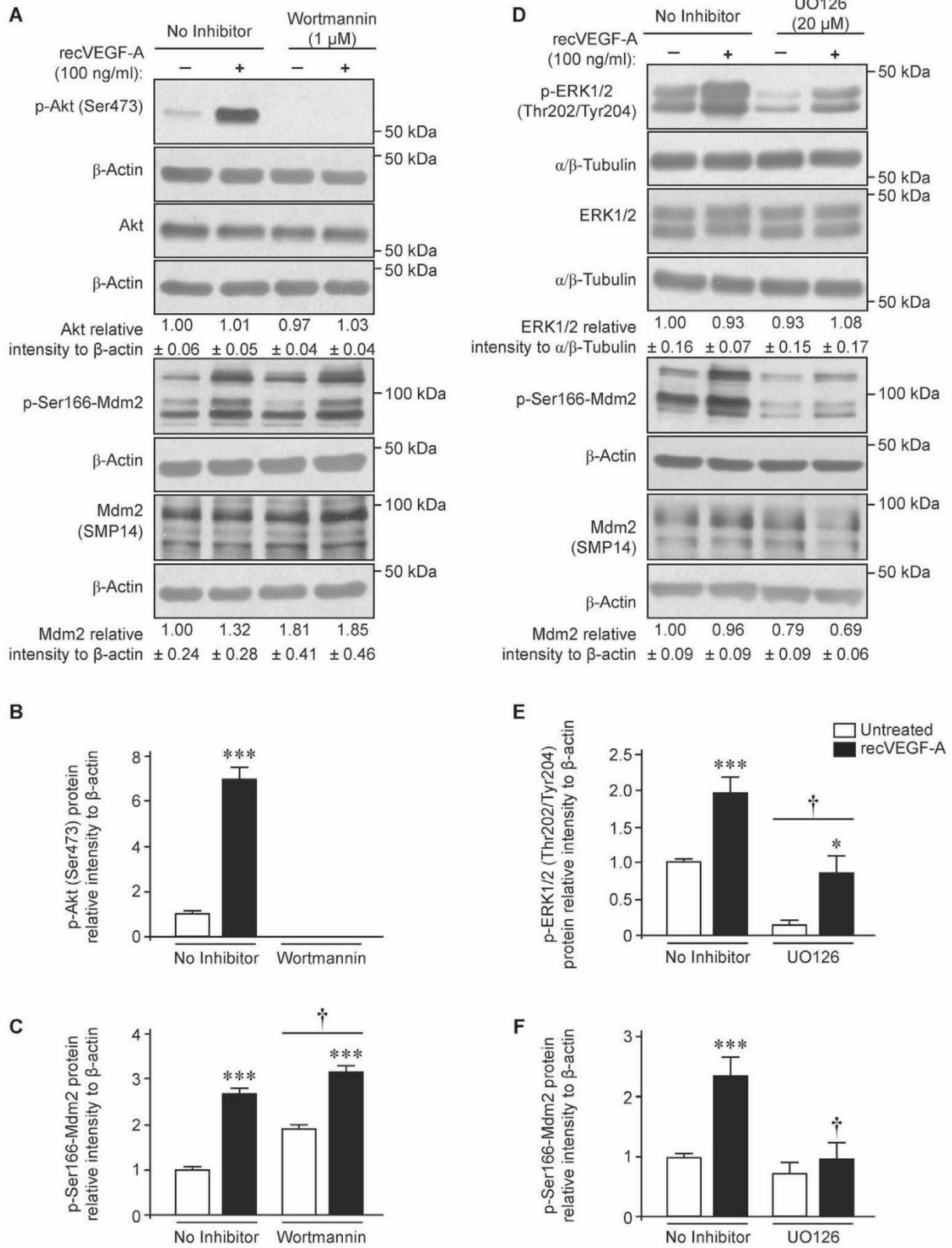
UO126 treatment. In A-F,  $\beta$ -actin or  $\alpha/\beta$ -tubulin was used as loading controls. Data are means  $\pm$  SEM (n = 2 samples per condition, repeated in 3 independent experiments). 2-way ANOVA and Bonferroni post hoc tests indicate; in B, \*\*\*  $P \leq 0.001$  vs. all other conditions; in C, \*\*\*  $P \leq 0.001$  vs. untreated within each respective condition, †  $P \leq 0.01$  vs. no inhibitor conditions; in E, \*\*\* $P \leq 0.001$  and \*  $P \leq 0.05$  vs. each respective untreated group, †  $P \leq 0.01$  vs. no inhibitor conditions; in F, \*\*\*  $P \leq 0.001$  vs. untreated, †  $P \leq 0.001$  vs. VEGF-A treated in the no inhibitor condition.

Figure 8.3 The p90RSK inhibitors FMK and BI-D1870 ablate the response of p-Ser166-Mdm2 to VEGF-A stimulation in primary human endothelial cells. Immunoblotting and densitometry analysis were performed on cell lysates of HDMECs collected after treatment with or without recVEGF-A (100 ng/ml) in the presence or absence of the p90RSK inhibitors FMK (25  $\mu$ M) or BI-D1870 (10  $\mu$ M), respectively. (A,B) Representative immunoblots and densitometry analysis for p-Ser166-Mdm2 and Mdm2 in response to recVEGF-A and FMK treatment. (C,D) Representative immunoblots and densitometry analysis for p-Ser166-Mdm2 and Mdm2 in response to recVEGF-A and BI-D1870 treatment. In A-D,  $\beta$ -actin or  $\alpha/\beta$ -tubulin were used as loading controls. Data are means  $\pm$  SEM (n = 2 samples per condition, repeated in 3 independent experiments). 2-way ANOVA and Bonferroni post hoc tests indicate \*\*\*  $P \leq 0.001$  vs. untreated; \*\*  $P \leq 0.01$  vs. untreated; †  $P \leq 0.01$  vs. VEGF-A treated in the no inhibitor condition.

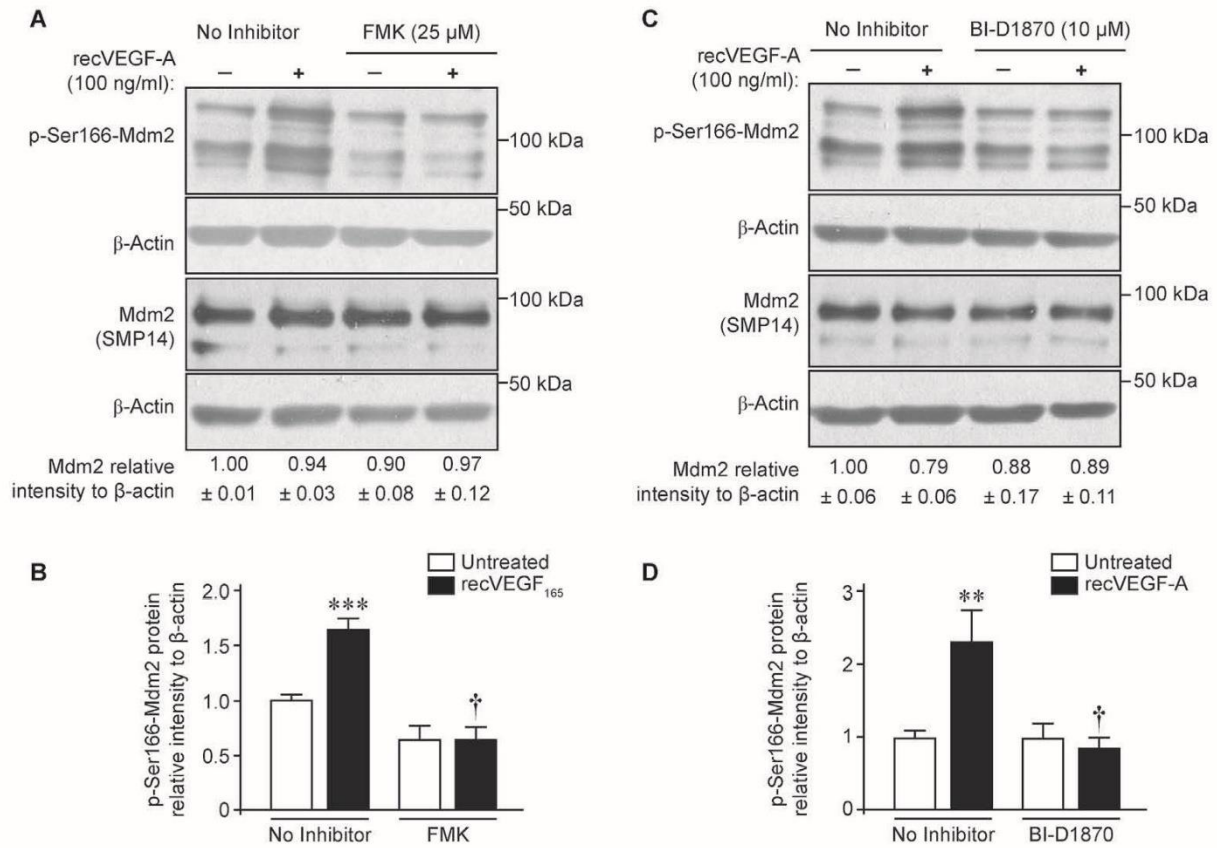
**Figure 8.1**



**Figure 8.2**



**Figure 8.3**





## 9. ACADEMIC RESEARCH PAPER 4

**Hyperglycemia strongly correlates with skeletal muscle capillary regression and is associated with alterations in the murine double minute-2/foxO1/thrombospondin-1 pathway in type 1 diabetic BioBreeding rats.**

**Julian Aiken**, Erin R. Mandel, Michael C. Riddell and Olivier Birot

\*submitted to *PLoS ONE* journal – submission ID: PONE-D-18-06901

### Author contributions

Julian Aiken:

- Contributed to formulate and design all experiments
- Performed all western blotting
- Performed all statistical analysis (i.e. correlations)
- Contributed to all data analysis
- Contributed in the preparation of the manuscript and all figures

### Scientific context:

- Our laboratory has previously shown that Mdm2 protein is significantly reduced in Zucker diabetic fatty (ZDF) rats, a model of type-2 diabetes
- Other studies suggest that type 1 diabetes (T1D) leads to capillary regression and the alteration of key angio-adaptive molecules in the skeletal muscle [180,187]
- I have demonstrated that Mdm2 levels are decreased in streptozotocin-induced T1D in rats, corresponding with skeletal muscle capillary regression (see appendix for academic research paper 5 [180])

- It is yet to be determined how T1D-induced alterations in Mdm2 could potentially lead to shifts in the angiogenic balance as explained by changes in key Mdm2-dependent targets, FoxO1, TSP-1 and VEGF-A
- Previous studies investigating muscle capillaries in type 1 diabetes have exclusively utilized rodent models in which T1D is induced through systemic streptozotocin injection

**Research aims:**

- Determine whether hyperglycemia resulting from spontaneous T1D development in BioBreeding (BB) diabetic rats leads to skeletal muscle capillary regression
- Investigate whether T1D leads to maladaptive expression of Mdm2 and its key targets VEGF-A, FoxO1, and TSP-1 in the skeletal muscle of diabetic animals
- Compare between soleus and plantaris, two metabolically distinct skeletal muscles

**Summary of main findings:**

- T1D resulted in significant capillary regression in both the soleus and plantaris muscles
- This capillary impairment was significantly correlated with the level of glycemic control in diabetic animals
- Mdm2 protein was found to be decreased in the diabetic muscle, corresponding with increased FoxO1 and TSP-1 protein levels
- Despite no change or even elevated VEGF-A protein, the VEGF-A/TSP-1 ratio and VEGFR2 protein levels were significantly less in the diabetic animals

- Significant correlations were observed between changes in blood glucose and Mdm2, TSP-1, VEGFR2 and the capillary-to-fiber ratio
- Alterations in Mdm2 and TSP-1 were inversely and significantly correlated

**Hyperglycemia strongly correlates with skeletal muscle capillary regression and is associated with alterations in the murine double minute-2/foxO1/thrombospondin-1 pathway in type 1 diabetic BioBreeding rats**

Submitted to *PLoS ONE* journal – submission ID: PONE-D-18-06901

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## **Abstract**

Type 1 diabetes (T1D) is an autoimmune disease that causes a loss in insulin production that can have deleterious effects on skeletal muscle and its microvasculature. Studies that have investigated alterations in skeletal muscle capillarization in T1D are limited and mainly based on the toxin model of streptozotocin-injection in rodents. Our laboratory has recently identified Murine Double Minute-2 (Mdm2) as a master regulator of skeletal muscle capillarization by controlling expression levels of two key molecular actors of the angio-adaptive process: the pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A) and the anti-angiogenic Thrombospondin-1 (TSP-1). The effect of T1D on these key regulators of muscle capillarization are unclear. Here, we show for the first time that the diabetic-prone BioBreeding (DPBB) rat, a rodent model of autoimmune T1D, undergoes capillary regression and altered protein expression of key angio-adaptive regulators in both plantaris and soleus skeletal muscles. We demonstrate that Mdm2 protein levels are significantly decreased in the skeletal muscles of DPBB rats, coinciding with elevated protein levels of TSP-1 and its transcription factor Forkhead Box O1 (FoxO1). Significant capillary regression was observed in T1D rats to similar extents in the soleus and plantaris muscles. Interestingly, manipulating blood glucose control in these animals, by using insulin implants, was significantly and positively correlated with the loss of capillaries, the reduction in Mdm2 expression, and with the elevations in TSP-1. VEGF-A protein levels were unaltered or even increased in diabetic animals, however we observed significantly less VEGF receptor-2 abundance. Furthermore, the VEGF-A / TSP-1 ratio, a good indicator of the skeletal muscle angio-adaptive environment, was significantly decreased in T1D muscle. Altogether, our results suggest that the Mdm2-FoxO1-TSP-1 signaling pathway plays an important role in angio-regulation of the skeletal muscle in the pathophysiological context of T1D.

## Introduction

Type 1 diabetes (T1D) is an autoimmune disease that results in the destruction of pancreatic  $\beta$ -cells and the loss of endogenous insulin production, leading to hyperglycemia and reliance on exogenous insulin administration [1]. Even with exogenous insulin therapy, control is often imperfect in T1D, with extensive lifetime exposure to hyperglycemia. Control is particularly poor in youth and young adults and there appears to be a legacy effect of poor control on clinical complications later in life [2]. Hyperglycemia is a major risk-factor for endothelial cell dysfunction, both directly and indirectly [1]. Consequently, T1D is associated with a number of microvascular complications affecting our smallest blood vessels, the capillaries. We and others have previously observed in rodent models of T1D a loss of capillaries in skeletal muscle [3,4]. The skeletal muscle tissue is paramount for a variety of functions ranging from locomotion to the metabolic regulation of glycemia. Indeed, skeletal muscle is the major site of glucose uptake in the postprandial state in healthy humans, accounting for ~80% of the body's insulin-mediated glucose disposal [5]. With muscular exercise, the rate of glucose uptake into muscle increases up to 50-fold [6,7]. Since capillaries are responsible for the delivery of oxygen and nutrients to skeletal myofibers, the capillary network appears as a key determinant of overall muscle functionality and it is crucial that the equipment in capillaries perfectly matches the metabolic needs of active myofibers. For endogenous (i.e. pancreatic) or exogenous (i.e. via needle or pump) insulin to promote glucose uptake into muscle, it must first distribute among the muscle capillaries, pass the endothelial barrier and diffuse through the interstitial space and bind with its receptor on the myocyte. A failure to deliver insulin to the target location (i.e. muscle) is now regarded as another reason for reduced insulin sensitivity [8], but the role of the capillaries in this process in T1D is not completely understood.

With a remarkable plasticity, the capillary network can grow, regress, or simply keep maintained in order to adapt to the myofibers' needs. Such skeletal muscle angio-adaptation is tightly regulated by a dynamic balance between pro- and anti-angiogenic molecules [9]. We have previously identified the E3 ubiquitin ligase Murine Double Minute-2 (Mdm2) as indispensable for skeletal muscle capillary maintenance, physiological capillary growth (i.e. angiogenesis) and the pro-angiogenic activity of endothelial cells (i.e. cell migration) [10-12]. We have also shown that Mdm2 could be considered a master regulator of skeletal muscle angio-adaptation as it can modulate both sides of the angio-adaptive balance, most notably, through the regulation of the pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A) and the anti-angiogenic Thrombospondin-1 (TSP-1) [10,12]. In the context of diabetes, our laboratory has recently shown that Mdm2 protein levels were significantly lowered in skeletal muscles of type 1 and 2 diabetic animals, coinciding with muscle capillary regression [4,10].

Several studies suggest altered muscle capillarization and microvascular function in humans living with T1D. It has been observed, using human and animal models, that T1D prevents exercise training-induced angiogenesis [13], unmatched capillary perfusion alongside myofiber hypertrophy in response to exercise training [14], an enlargement of the capillary basement membrane hindering perfusion and vascular elasticity [15], and reduced peripheral skeletal muscle perfusion both at rest and after ischemia [16]. Furthermore, diabetes has been shown to impair the development of coronary collateral vessels [17]. Although very limited in number, previous studies that have investigated the impact of T1D on skeletal muscle molecular angio-adaptation have exclusively utilized chemically-induced diabetes through streptozotocin (STZ) injection in rodents. STZ is a highly toxic alkylating agent that leads to hyperglycemia by accumulating in pancreatic  $\beta$ -cells through the glucose transporter-2 (GLUT-2) causing DNA alkylation and  $\beta$ -cell

death [18,19]. The onset of overt hyperglycemia is rapid, occurring in as little as 48-72 hours following STZ injection [18,20]. The systemic administration of STZ therefore represents an easy and cost-effective diabetes-inducing approach that is similar to the severe insulinopenia that occurs in untreated T1D. However, it raises questions regarding the toxicity of the substance in other tissues [21]. Indeed, the uptake of STZ by GLUT-2 is not specific to pancreatic  $\beta$ -cells, therefore being potentially toxic to other tissues and organs, such as the kidney and liver, potentially leading to confounding experimental results [18,21,22]. In cell culture and in vivo, STZ directly causes muscle protein ubiquitination, cell cycle arrest and reduced cell proliferation rates, independent of its impact on glycemia [23]. Alternatively, BioBreeding (BB) diabetic rats spontaneously develop T1D between 50-90 days of age, around the time of puberty, and present characteristics that closely resemble those of human patients with T1D, including exogenous insulin dependency, hyperglycemia, polydipsia, polyuria, and ketoacidosis [24-28]. As is the case with human T1D patients, the onset of diabetes in the BB rat is characterized by the infiltration of immune cells in the islets of Langerhans (insulinitis) [28], preceding overt diabetes by two to three weeks. Subsequently,  $\beta$ -cells of the pancreas are selectively destroyed resulting in a severe type 1 diabetic phenotype. BB diabetic rats are classified as either diabetic-prone (DPBB) or diabetic-resistant (DRBB), the latter serving as a control group that have the same diabetes-susceptible genes but do not spontaneously develop T1D in viral-free conditions. In comparing the timeline of diabetes onset between BB rats and STZ-induction, the BB rat represents a chronic pathological state compared to the rapid development of diabetes induced by STZ. The selection of the BB rat model for our investigation is relevant since the skeletal muscle and its associated microvasculature undergo considerable growth and development during the age of onset in the BB rat, and are likely impacted by the level of glycemic control achieved [29].



To date, it is still unclear how, and to which extent, T1D could affect the microcirculation in skeletal muscle, especially in terms of capillarization. Here, we sought to determine whether capillary regression occurs in skeletal muscles from diabetic prone BB rats in order to more closely mimic the installation of type 1 diabetes seen in humans. We aimed to identify potential diabetes-induced alterations in key angio-regulatory molecules such as Mdm2, VEGF-A and TSP-1. To our knowledge, this is the first study to investigate skeletal muscle angio-regulation in the BB diabetic rat. Understanding deleterious adaptations that occur in skeletal muscle as a result of T1D is of great importance for both the treatment and prevention of diabetes-associated vascular pathology.

## **Materials and methods**

*Animal model.* This study was approved by the York University Animal Care Committee and was conducted in accordance with the Canadian Council for Animal Care Guidelines. Animals were bred at Sunnybrook Research Institute, Toronto, Canada, courtesy of Dr. Philippe Poussier (protocol #520). BioBreeding diabetes-prone (DPBB) rats and age-matched diabetes-resistant (DRBB) rats (n = 20 & 22 respectively) were housed under temperature-controlled conditions in a 12-hour light-dark cycle with *ad libitum* access to a standard rodent chow diet and water. DPBB animals typically become diabetic (blood glucose [BG]  $\geq$  15mM) at a young age (i.e. between 50 & 90 days post birth) and require insulin treatment to survive. Our blood glucose threshold for the determination of diabetes is in line with standard guidelines [30]. After diabetes was established, the animals' blood sugar was maintained between 12-20 mM to simulate the fair to poor glycemic control that is generally observed in youth living with the disease [31] by using subcutaneous insulin pellets (Linchen Canada) for a period of two weeks. Briefly, under inhaled isoflurane anesthetic a 0.5cm subcutaneous incision, just below the neck, was made and half to a full insulin pellet was implanted (LinShin, Canada Inc., Toronto, Ontario, Canada), with a full pellet releasing

about 2U of insulin/24hours for up to 40 days. The incision wound was closed and treated with topical antibiotics and pain cream (Flamazine, Smith & Nephew Canada) prior to being placed back in their cage.

*Glucose measurement.* Whole blood glucose levels were measured daily using an AlphaTRAK2 glucose meter (Abbott Diabetes Care Inc., Alameda CA) from a sterile saphenous vein bleed. Blood glucose values reported here were recorded just prior to skeletal muscle harvest.

*Skeletal muscle tissue isolation.* Two weeks after insulin pellet implantation, animals were anaesthetized with inhaled isoflurane. Soleus and plantaris muscles were removed, weighted and immediately flash frozen in liquid nitrogen (protein measurements) or frozen in liquid nitrogen-cooled isopentane for histology. All samples were stored at (-80°C) until analysis.

*Muscle capillarization analysis.* Transverse 10µm cryosections were obtained from the mid-belly of plantaris and soleus muscles from DRBB and DPBB rats using a cryostat set to -18°C (Leica CM1860) as previously described [10]. Capillaries were visualized after a brief fixation of cryosections in cold acetone followed by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (FAST BCIP/NBT; cat. no. B5655-25TAB; Sigma-Aldrich, Oakville, ON, Canada) for 45 minutes at 37°C to stain for alkaline phosphatase activity. Microscope slides were mounted with VectaMount (cat. no. H-5501; Vector Laboratories Inc., Burlingame, CA, USA). Images were acquired on an Arcturus PixCell II inverted microscope (serial no. 0573; Arcturus Engineering, Mountain View, CA, USA) at x10 magnification and captured using QCapture software. For each muscle, the capillary-to-fiber ratio was determined after counting capillaries and myofibers in 6 independent images of a muscle cross section. Because the plantaris muscle is not homogeneous in its fiber-type composition, counting areas were chosen so

they cover all the muscle cross section. Capillary-to-fiber ratio is calculated by dividing the number of capillaries in each image by the number of myofibers.

*Western blotting.* Immunoblotting was carried out on protein extracts from the plantaris and soleus muscles obtained from DRBB and DPBB rats, respectively, as previously described [10,11]. Protein concentrations for each sample were determined by the bicinchoninic acid (BCA) assay, and denatured samples (30 µg/sample) were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and blotted onto nitrocellulose membranes (Whatman, BA95, Sigma-Aldrich, Oakville, Ontario, Canada). Quality of the transfer was confirmed by Ponceau S red staining. After blocking with 5% fat-free milk at room temperature for 45 min, the blots were probed overnight at 4°C with the following primary antibodies: antibody against Mdm2 was a non-commercial supernatant from the hybridoma (clone 2A10, previously described in [32]); antibody against FoxO1 was from Cell Signaling Technology (clone C29H4, #2880, Beverly, MA, USA); antibody against TSP-1 was from Invitrogen (clone A6.1, #MA5-13398, Burlington, ON, Canada); antibody against VEGF-A was from Millipore (clone VG1, #05-1117, Etobicoke, ON, Canada); antibodies against β-actin (#sc-47778) and VEGFR2 (clone D-8, #sc-393163) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After incubation with secondary antibodies HRP-linked anti-mouse (#P0260; Dako, Carpinteria, CA, USA) or HRP-linked anti-rabbit antibody (#7074; Cell Signaling), proteins were visualized with enhanced chemiluminescence (Millipore) on X-ray film (CL-XPosure Film, #34090, Thermo Scientific, Rockford, IL, USA). Blots were analyzed with Carestream software.

*Statistical analysis.* Statistical analyses were performed using Prism5 software (GraphPad, San Diego, CA, USA). Unpaired, two-tailed t-tests were performed for blood glucose, capillary-to-fiber ratio, and all western blot analyses. Correlation analyses between variables were performed

with nonparametric 2-tailed Pearson correlation with determination of Pearson  $r$  and  $r^2$ .  $P \leq 0.05$  was considered to be statistically significant.

## Results

*Type 1 diabetes leads to hyperglycemia and skeletal muscle capillary regression.* As expected, DPBB rats spontaneously develop type 1 diabetes (T1D), reflected by significantly higher blood glucose levels (+259%) measured at the time of muscle collection compared to DRBB animals (Fig 9.1A; DRBB,  $6.58 \pm 0.17$  vs. DPBB rats,  $23.64 \pm 1.39$  mmol/L;  $P \leq 0.001$ ). T1D leads to capillary regression in both plantaris (-15%) and soleus (-21%) muscles of DPBB rats as evidenced by measuring the capillary-to-fiber ratio (Fig 9.1B; DRBB,  $1.68 \pm 0.039$  vs. DPBB,  $1.43 \pm 0.049$  in plantaris; DRBB,  $1.90 \pm 0.045$  vs. DPBB,  $1.52 \pm 0.063$  in soleus;  $P \leq 0.001$ ). The level of skeletal muscle capillarization had a significant inverse correlation with blood glucose levels in both muscles analyzed (Fig 9.1C;  $r = -0.327$ ,  $P < 0.05$  in plantaris;  $r = -0.479$ ,  $P < 0.01$  in soleus), suggesting that hyperglycemia is a strong predictor of capillary regression.

*Type 1 diabetes results in lowered Mdm2 and increased anti-angiogenic FoxO1 and TSP-1 protein levels in skeletal muscle.* As DPBB rats displayed a significant reduction in the number of skeletal muscle capillaries, with the severity of impairment related at least in part to the level of glucose control, we next aimed to determine which key angio-regulatory factors could be involved in the observed regression of capillaries. Diabetic DPBB rats had a significantly reduced expression of Mdm2 protein in both the plantaris (-19%) and soleus (-15%) muscles (Fig 9.2A; DRBB,  $0.77 \pm 0.05$  vs. DPBB,  $0.62 \pm 0.04$  arbitrary units in plantaris; DRBB,  $0.74 \pm 0.04$  vs. DPBB,  $0.63 \pm 0.02$  arbitrary units in soleus;  $P < 0.05$ ). Alongside decreased levels of Mdm2 protein in diabetic muscles, DPBB rats had ~80% more FoxO1 protein in the plantaris muscle compared to controls (Fig 9.1B; DRBB,  $1.07 \pm 0.09$  vs. DPBB,  $1.92 \pm 0.3$  arbitrary units;  $P <$

0.05). Although not statistically significant, diabetic animals also tended to express more (~20%) FoxO1 protein in the soleus than what was observed in the control rodents (Fig 9.1B; DRBB,  $1.12 \pm 0.06$  vs. DPBB,  $1.31 \pm 0.09$  arbitrary units;  $P = 0.075$ ). Concomitant with the decrease in Mdm2 and the increase in FoxO1 expression, TSP-1 protein expression was drastically higher in skeletal muscles from diabetic DPBB rats compared to control rats (+172% and +67% in plantaris and soleus muscle, respectively) (Fig 9.1C; DRBB,  $0.32 \pm 0.04$  vs. DPBB,  $0.88 \pm 0.1$  arbitrary units in plantaris,  $P < 0.01$ ; DRBB,  $0.91 \pm 0.15$  vs. DPBB,  $1.51 \pm 0.13$  arbitrary units in soleus,  $P < 0.001$ ).

*Hyperglycaemia is correlated with decreases in Mdm2 and elevations in TSP-1 protein expression.* Correlation analyses were performed between blood glucose level, Mdm2, and TSP-1 protein expression in plantaris and soleus muscles from DPBB and DRBB rats. Interestingly, in the plantaris muscle, blood glucose levels were inversely correlated with Mdm2 protein expression (Fig 9.3A;  $r = -0.312$ ,  $P < 0.05$ ). A similar finding was observed in the soleus muscle, although this relationship was on the border of statistical significance (Fig 9.3A;  $r = -0.303$ ,  $P = 0.054$ ). In both the soleus and plantaris muscle, blood glucose was found to be positively correlated with TSP-1 protein levels (Fig 9.3B;  $r = 0.557$ ,  $P < 0.001$  in plantaris;  $r = 0.481$ ,  $P < 0.01$  in soleus). Furthermore, in both muscles, Mdm2 and TSP-1 protein expression were significantly, and inversely, correlated (Fig 9.3C;  $r = -0.335$ ,  $P < 0.05$  in plantaris;  $r = -0.424$ ,  $P < 0.01$  in soleus).

*The VEGF-A/TSP-1 ratio is drastically reduced in skeletal muscles of type 1 diabetic animals.* Interestingly, DPBB rats show no change (soleus) or even an increase (plantaris) in VEGF-A protein expression level compared to non-diabetic animals (Fig 9.4A; DRBB,  $0.37 \pm 0.02$  vs. DPBB,  $0.48 \pm 0.04$  arbitrary units in plantaris,  $P < 0.05$ ; DRBB,  $1.14 \pm 0.04$  vs. DPBB,  $1.12 \pm 0.04$  arbitrary units in soleus,  $P = 0.68$ ). We have previously described the VEGF-A/TSP-1 ratio as a good indicator of the angio-adaptive balance in rodent and human skeletal muscles

[9,10,12,33]. Here, and despite either no alteration (soleus) or even an increase (plantaris) in VEGF-A protein expression, the VEGF-A/TSP-1 ratio was strongly reduced in the plantaris (-52%) and soleus (-71%) muscles of type 1 diabetic rats compared to non-diabetic animals (Fig 9.4B; DRBB,  $1.52 \pm 0.2$  vs. DPBB,  $0.74 \pm 0.15$  in plantaris,  $P < 0.01$ ; DRBB,  $2.92 \pm 0.9$  vs. DPBB,  $0.87 \pm 0.09$  in soleus,  $P < 0.05$ ).

*VEGF receptor-2 protein levels are decreased in type 1 diabetic muscle.* In the soleus, a significant decrease (-32%) in Vascular Endothelial Growth Factor receptor-2 (VEGFR2) protein level was observed in DPBB rats compared to DRBB controls (Fig 9.5A; DRBB,  $0.35 \pm 0.03$  vs. DPBB,  $0.24 \pm 0.02$  arbitrary units,  $P < 0.01$ ). Although not statistically significant, VEGFR2 protein expression was reduced by ~30% in the plantaris of (Fig 9.5A; DRBB,  $0.16 \pm 0.02$  vs. DPBB,  $0.11 \pm 0.01$  arbitrary units,  $P = 0.06$ ). Interestingly, a significant and inverse correlation was found between VEGFR2 protein and blood glucose levels in the soleus (Fig 9.5B;  $r = -0.427$ ,  $P < 0.01$ ), while a near-significant correlation was also found in the plantaris (Fig 9.5B;  $r = -0.307$ ,  $P = 0.051$ ).

## **Discussion**

We showed here for the first time using the BioBreeding rodent model of T1D that in skeletal muscles from T1D rats poor blood glucose control is associated with capillary regression and alterations in protein expression of key angio-regulatory molecules. We performed our investigation in two muscles with distinct contractile and metabolic phenotypes: the fast-twitch and predominantly glycolytic plantaris and the slow-twitch oxidative soleus. As summarized in Figure 9.6, we propose that T1D led to similar levels of capillary regression in both muscle types likely as a result of decreased Mdm2 and VEGFR2 levels, and elevated FoxO1 and TSP-1 proteins. While the direction and general trend of alteration in angio-adaptive molecules was found to be

similar between the plantaris and soleus muscle, the magnitude of change differed, especially for FoxO1, TSP-1, and VEGFR2. This could be explained by phenotypic differences in contractile and metabolic properties of the respective muscles, potentially leading to muscle type-specific regulation of the measured proteins. Nonetheless, despite these differences, the appreciable end result of capillary loss was comparable between the soleus and plantaris. Remarkably, alterations in Mdm2 expression and the VEGF-A/TSP-1 ratio were also very similar between the two muscles with the ratio reflecting a shift of the angio-adaptive balance towards an anti-angiogenic environment in muscles from T1D animals. This is in line with our previous work demonstrating that Mdm2 is a central regulator of skeletal muscle angio-adaptation and that such a complex biological process must be studied with an integrative approach taking into consideration both pro- and anti-angiogenic signals [10,12,33].

It is well documented that T1D leads to an array of microvascular-related pathologies including retinopathy, neuropathy and nephropathy [34]. These three most common diabetic comorbidities typically arise through poor glycemic regulation leading to endothelial degeneration or (paradoxically) hyper-vascularization, alterations in vascular permeability, and changes in blood pressure [1]. However, very little research exists examining the molecular actors regulating skeletal muscle microvasculature in type 1 diabetes. In addition, those limited existing studies were based on STZ injection as a means to induce diabetes in rodent models. STZ treatment leads to the rapid destruction of pancreatic  $\beta$ -cells and subsequent hyperglycemia, but also can promote direct damage to other organs and tissues including skeletal muscle [23]. Alternatively, BB rats represent a chronic condition with the onset of diabetes occurring pathophysiologically as a result of spontaneous autoimmune-mediated destruction of pancreatic  $\beta$ -cells, similar to the disease progression in humans. In consequence, STZ-induced diabetes may represent a reasonable but

somewhat flawed model for the investigation of new insulin formulations and transplantation experiments, while the use of BB rats could be more appropriate for understanding the underlying mechanisms of T1D-mediated alterations including those affecting skeletal muscle angio-adaptation [21]. In our study, the onset of T1D in the BB rat model indeed led to significant capillary regression in the skeletal muscle tissue (within a one to two weeks period), characterized by a true loss of existing capillaries as evidenced by the decreased capillary-to-fiber ratio. Conversely to capillary density, which can be affected by capillary loss but also by any change in the size of myofibers, the capillary-to-fiber ratio represents the gold standard for assessing true muscle angiogenesis or capillary regression. Here, we observed that the level of dysglycaemia (~2-3 fold higher values than normal despite exogenous insulin therapy) were significantly correlated with alterations in the capillary-to-fiber ratio.

We next aimed to explore key angio-adaptive molecular pathways that could be involved in such T1D-induced capillary regression. Our laboratory has recently identified Mdm2 as essential for the maintenance and plasticity of skeletal muscle microvasculature. In a pioneering study using transgenic mice expressing about 60% less Mdm2 compared to their wild-type littermates, Mdm2 deficiency resulted in a 20% reduction in skeletal muscle capillarization and fully blunted the exercise-induced angiogenic response [10]. We also showed that Mdm2 and its human analog Hdm2 were strongly associated with the level of skeletal muscle capillarization and endothelial markers in both rodents and humans [10,11]. The mechanisms through which reduced Mdm2 levels can lead to capillary regression are complex, as Mdm2 can be considered a ‘master regulator’ of the angio-adaptive balance affecting both pro- and anti-angiogenic factors (i.e. VEGF-A and TSP-1). VEGF-A is the most widely studied pro-angiogenic factor, promoting endothelial cell survival, proliferation, and migration [12,35-37]. Through the use of transgenic



animal models where VEGF-A had been deleted specifically in myofibers, we and others have shown that VEGF-A deficiency leads to significant capillary regression in skeletal muscle tissue [12,38]. It has been suggested that Mdm2 could stabilize Hypoxia-Inducible Factor-1 $\alpha$  (HIF-1 $\alpha$ ), a transcription factor for VEGF-A, thereby promoting VEGF-A expression [39-42]. Aside from the HIF-1 $\alpha$ /VEGF-A pathway, we have also shown that Mdm2 can directly bind to and negatively regulate the activity of the transcription factor FoxO1 and thus the expression of its downstream target TSP-1, a potent anti-angiogenic factor in skeletal muscle [10,12,43,44]. Interestingly, it has also been demonstrated that overexpression of FoxO1 in endothelial cells led to a decrease in VEGF-A mRNA levels suggesting FoxO1 could regulate not only TSP-1, but rather both sides of angio-adaptive balance (i.e. VEGF-A and TSP-1) [45].

In the context of diabetes, we have previously reported that Mdm2 protein expression was altered in skeletal muscle from Zucker Diabetic Fatty (ZDF) rats, a common model of obesity and type 2 diabetes. In ZDF plantaris muscles, Mdm2 was significantly lowered and a concomitant increase in FoxO1 protein levels was observed [10]. More recently, we reported a significant decrease in Mdm2 protein levels in both the tibialis anterior and soleus muscles of STZ-treated type 1 diabetic rats [4]. In the present study, we observed a decreased Mdm2 protein level in both soleus and plantaris muscles of DPBB rats. Taken together, our laboratory has investigated three separate models of diabetes (including both type 1 and type 2) in which skeletal muscle capillary regression has occurred. In each model, a significant reduction in Mdm2 protein levels was detected. The observed reductions in Mdm2 could potentially explain the loss of capillaries in type 1 diabetic animals through Mdm2-dependent regulation of VEGF-A, FoxO1 and TSP-1.

It is of vital importance to take into account both sides of the angio-adaptive balance when attempting to appreciate the overall effect of physiological and pathological stimuli on the skeletal

muscle microvasculature. As an important pro-angiogenic myokine, VEGF-A binds to receptors, and VEGFR2 plays a key role in mediating at the intracellular level the VEGF-A pro-angiogenic signal [46]. A previous study from Kivelä and colleagues [3] demonstrated that T1D induction by STZ injection significantly decreased mRNA levels of VEGF-A and VEGFR2 in the mouse calf muscle complex (gastrocnemius, soleus and plantaris). In accordance with VEGF-A mRNA expression, VEGF-A protein levels were significantly lower at both 3 and 5 weeks following the onset of diabetes. Similar to their findings, we show here reductions in VEGFR2 protein expression in diabetic muscles. However, in our study VEGF-A protein levels remained either unchanged (soleus) or even elevated (plantaris) in DPBB rats. Kivelä et al. [3] also show that TSP-1 mRNA was significantly higher in diabetic mice compared to healthy controls, yet did not measure TSP-1 at the protein level. Here, we show that T1D leads to significant elevations in both FoxO1 and TSP-1 protein expression, and that Mdm2 and TSP-1 protein expression (the respective start and end point of the Mdm2-FoxO1-TSP-1 signaling axis) were significantly and inversely correlated. We have recently established that the VEGF-A to TSP-1 ratio is a good indication of the overall angiogenic environment [9,10,12,33]. In both soleus and plantaris, this ratio was dramatically decreased regardless of VEGF-A protein levels, reflecting an anti-angiogenic microenvironment in the skeletal muscle tissue. In addition, previous studies demonstrated that TSP-1 can negatively regulate VEGFR2 through inhibition of VEGF-A-dependent VEGFR2 phosphorylation and activation in microvascular endothelial cells [47,48]. Based on these findings, an intriguing hypothesis could be made that the observed elevations in TSP-1 might inhibit VEGFR2 sensitivity to VEGF-A in diabetic muscles, concomitantly with an already diminished VEGFR2 protein abundance. Further investigation would be needed to elucidate this potentially novel regulatory relationship in T1D.

Our findings that some capillary regression occurred in T1D rats despite unchanged or increased VEGF-A expression levels is not surprising. Using hindlimb unloading as a well-established rodent model for muscle atrophy, we demonstrated that plantaris and soleus muscles, despite similar levels of myofiber atrophy, had entirely different angio-adaptive responses [33]. In the plantaris, where capillarization was maintained, VEGF-A levels were found to be increased. In contrast, capillary regression occurred in the soleus muscle despite no decrease in VEGF-A but concomitantly to a strong increase in TSP-1 and reduction in VEGFR2. Taken together, it appears that anti-angiogenic factors (i.e. TSP-1) are equally important, if not more influential, than their pro-angiogenic counterparts in regulating skeletal muscle angio-adaptation to physiological as well as pathological conditions such as type 1 and type 2 diabetes.

Interestingly, when focusing only on diabetic DPBB rats, the correlation analyses performed between blood glucose, capillary-to-fiber ratio, Mdm2, TSP-1 and VEGFR2 indicate that the DPBB rats that had lower blood glucose (i.e. 20 mmol/L) had similar magnitudes of angio-adaptive alterations as the DPBB rats with highest blood glucose levels. This suggests that even the lower-end of hyperglycemia level in our model could be sufficient to initiate a shift in the angio-adaptive balance leading to capillary loss in the muscle. The notion of such a hyperglycemic ‘threshold’ for changes at the molecular level leading to capillary regression is intriguing, and definitely warrants future research.

Here, we identified a novel relationship whereby the magnitude of capillary regression in skeletal muscle may be predicted by the level of glycemic control in type 1 diabetes. This relationship between muscle capillarization and blood glucose level is of importance since reduced capillarization may exacerbate poor glycemic regulation in patients with diabetes, as capillaries are essential for insulin delivery and provision of glucose for uptake by myofibers. The loss of

capillaries in response to diabetic dysglycemia may be preceded by pathological alterations in angio-regulatory factors that are sensitive to glucose control, in particular Mdm2 whose expression level is also directly correlated to blood glucose levels. We have previously demonstrated that Mdm2 is at the fork between pro-angiogenic VEGF-A and anti-angiogenic TSP-1 signals. Our present results suggest that Mdm2-FoxO1-TSP-1 signaling may represent a strong molecular determinant of this pathological adaptation of capillary regression in type 1 diabetic skeletal muscle. Our findings provide merit for future studies investigating the Mdm2 signaling axis as related to capillary regulation in the skeletal muscle of humans living with type 1 diabetes.

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## Figure legends

Figure 9.1. Type 1 diabetes in BB diabetes-prone rats results in hyperglycemia and decreased skeletal muscle capillarization. A) Blood glucose levels (mmol/L) in diabetes-resistant (DRBB) and -prone (DPBB) rats (n = 20 animals per group). The dashed line corresponding to 15mM blood glucose depicts the threshold where animals were considered diabetic. B) Top: Capillary staining in skeletal muscle cross-sections from DRBB and DPBB rat plantaris and soleus muscles (Scale bar = 100µm; x10 view). Bottom: Determination of the capillary-to-fiber ratio. Data are means ± SEM (n = 20-22 animals per group). Unpaired two-tailed t-tests indicate \*\*\* $P \leq 0.001$  vs. DRBB rats. C) Correlation analysis between blood glucose and capillary-to-fiber ratio in the animals used in A-B. Blue circles and red squares represent data points for DRBB and DPBB rats, respectively.

Figure 9.2. Type 1 diabetes alters protein expression levels of Mdm2, FoxO1 and TSP-1 in rat skeletal muscles. Representative immunoblots and densitometry analyses of protein expression are shown for Murine Double Minute-2 (Mdm2) (A), Forkhead Box O1(FoxO1) (B), and Thrombospondin-1 (TSP-1) (C) in plantaris and soleus muscles from type 1 diabetic rats (DPBB) and their controls (DRBB).  $\beta$ -actin was used as a loading control. Data are means ± SEM (n = 20-22 animals per group). Unpaired two-tailed t-tests indicate \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$  and \* $P \leq 0.05$  vs. DRBB rats.

Figure 9.3. Blood glucose levels are strongly correlated with Mdm2 and TSP-1 protein expression in skeletal muscle. Correlation analysis between blood glucose and Mdm2 protein (A), blood glucose and TSP-1 protein (B), and between Mdm2 and TSP-1 skeletal muscle protein levels (C) in the plantaris and soleus muscles of BB rats, respectively (n = 41-42 animals). Blue circles and red squares represent data points for DRBB and DPBB rats, respectively.

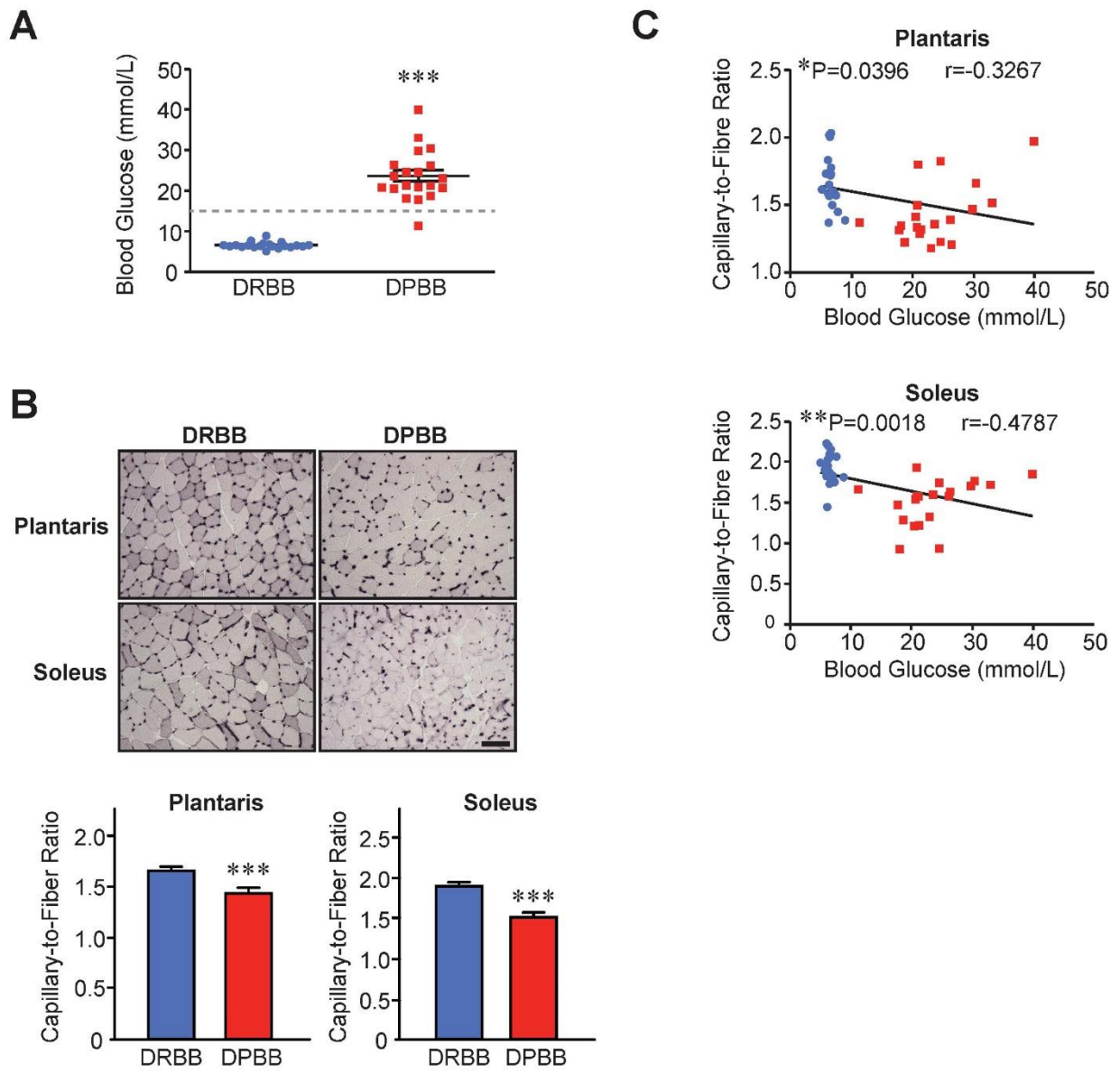
Figure 9.4. The ratio between TSP-1 and VEGF-A protein levels, and not VEGF-A alone, is a strong reflection of capillary regression in skeletal muscles from diabetic animals. (A) Representative immunoblots and densitometry analyses of VEGF-A protein expression and (B) the ratio of VEGF-A/TSP-1 protein expression in plantaris and soleus muscles from DRBB and DPBB rats, respectively.  $\beta$ -actin was used as a loading control. Data are means  $\pm$  SEM (n = 20-22 animals per group). Unpaired two-tailed t-tests indicate  $**P \leq 0.01$  and  $*P \leq 0.05$  vs. resistant rats.

Figure 9.5. VEGF receptor-2 protein expression is decreased in skeletal muscles of type 1 diabetic animals and is inversely correlated with blood glucose levels. (A) Representative immunoblots and densitometry analyses of VEGF receptor-2 (VEGFR2) protein expression in plantaris and soleus muscles from DRBB and DPBB rats.  $\beta$ -actin was used as a loading control. Data are means  $\pm$  SEM (n = 20-22 animals per group). Unpaired two-tailed t-tests indicate  $**P \leq 0.01$  vs. resistant rats. (B) Correlation analysis between blood glucose and VEGFR2 protein expression in the plantaris and soleus muscles of BB rats, respectively (n = 41 animals). Blue circles and red squares represent data points for DRBB and DPBB rats, respectively.

Figure 9.6. The BB rat model of type 1 diabetes results in hyperglycemia, skeletal muscle capillary regression, and altered angio-adaptive signaling. Type 1 diabetes in BB diabetes-prone rats results in significant elevation of blood glucose levels. This chronic hyperglycemic state induces angio-adaptive alterations observable at the tissue level such as capillary regression (capillary-to-fiber ratio, C/F) as well as at the molecular level with changes in the angio-adaptive signaling. Mdm2 serves as a negative regulator of FoxO1, therefore in the diabetic state, a down-regulation of Mdm2 could lead to elevated FoxO1 and its transcriptional target TSP-1, a critical anti-angiogenic factor in skeletal muscle tissue. VEGF-A however is not lowered in the skeletal muscle of diabetic animals, whereas the expression of its receptor-2 (VEGFR2) is significantly reduced. Interestingly,

several significant correlations were found between some of these parameters (blood glucose level, capillaries, molecular markers) measured individually.

**Figure 9.1**



**Figure 9.2**

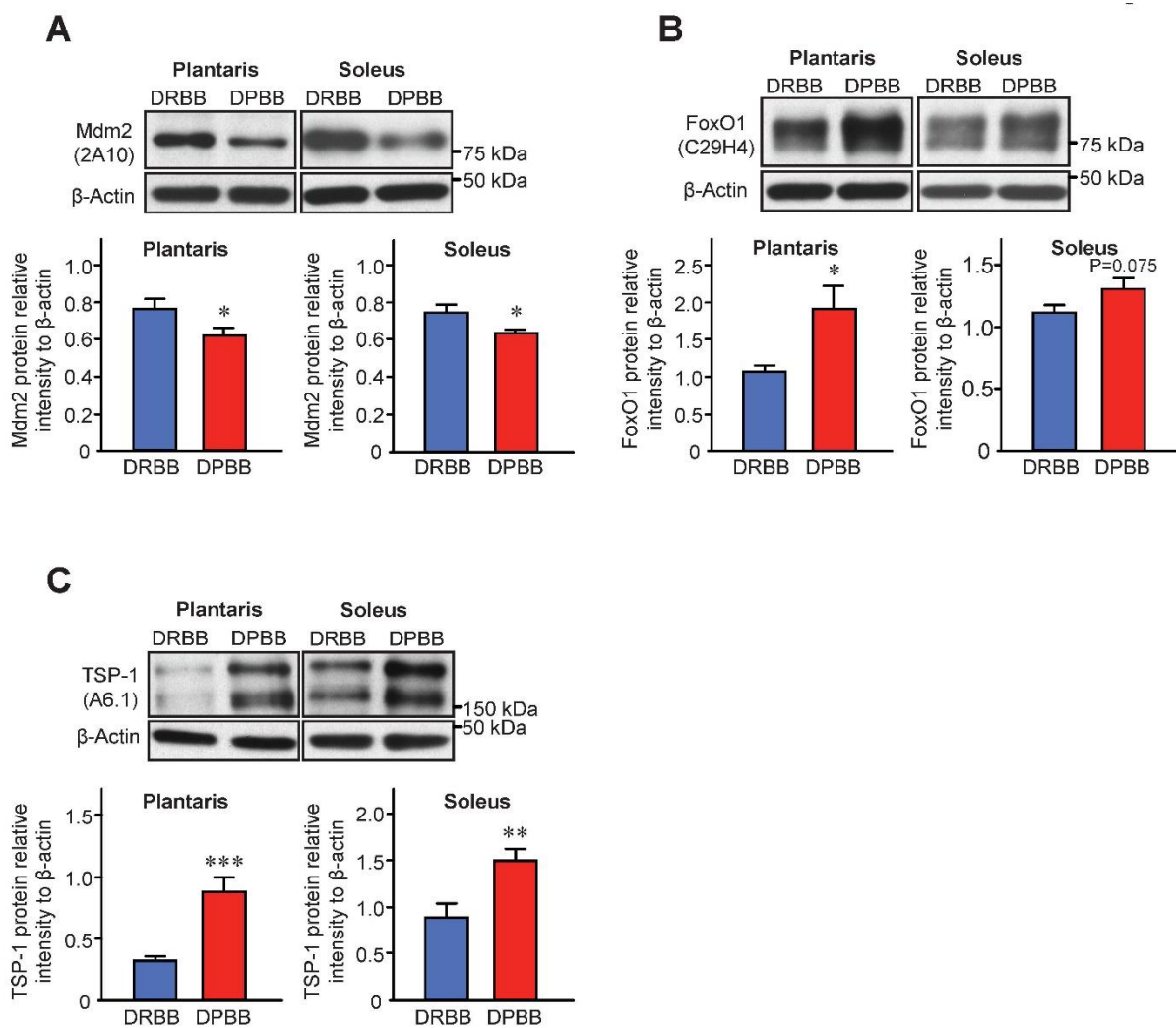




Figure 9.3

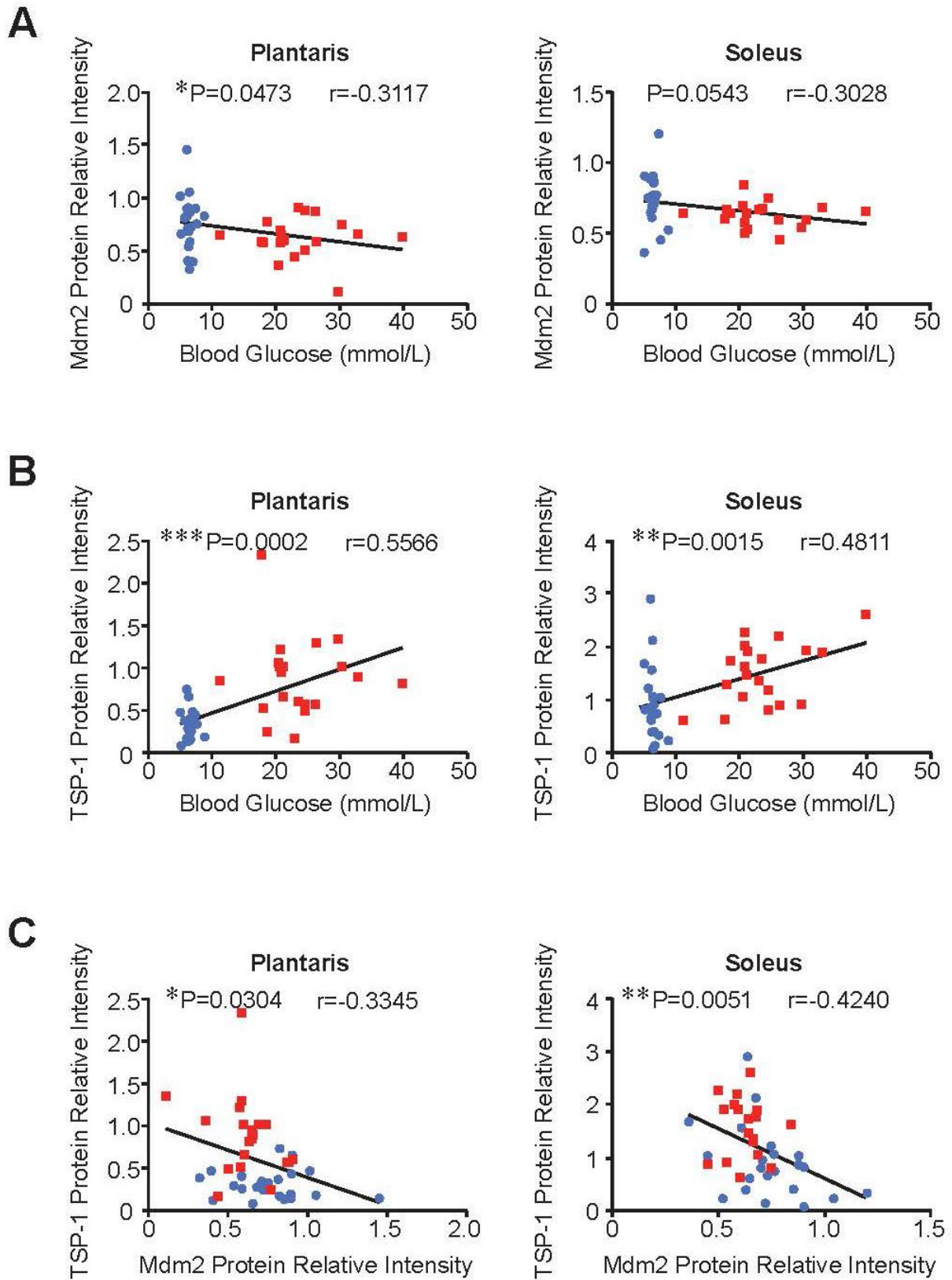


Figure 9.4

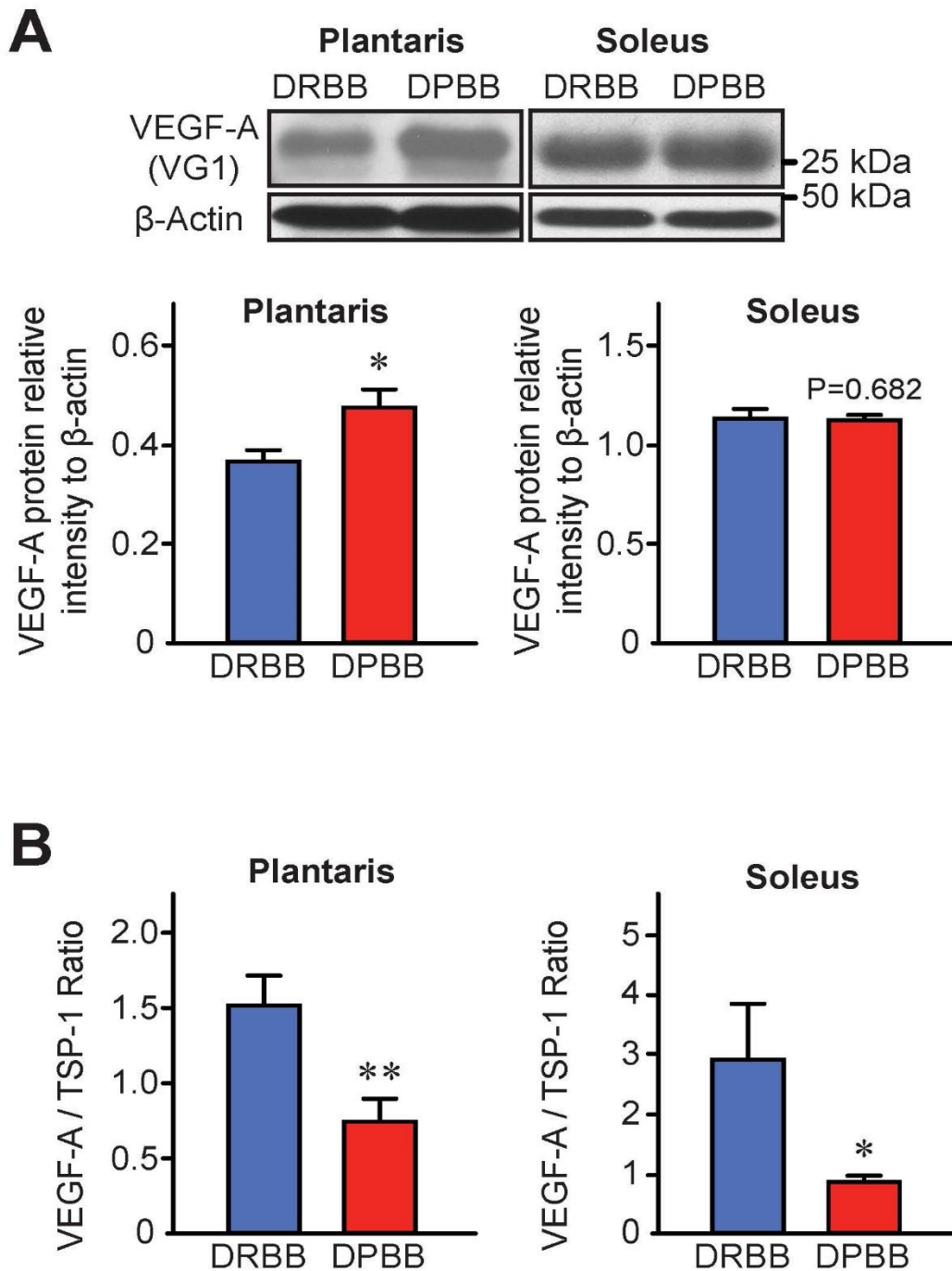
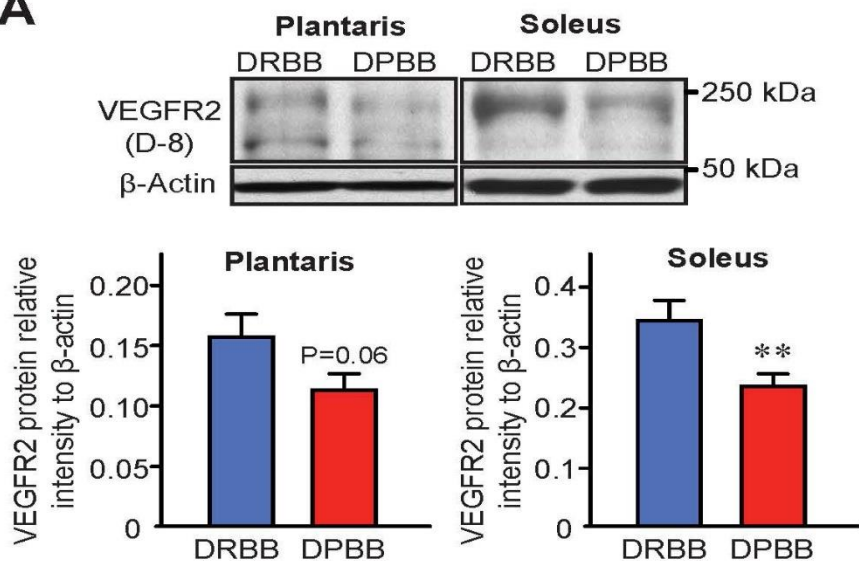


Figure 9.5

**A**



**B**

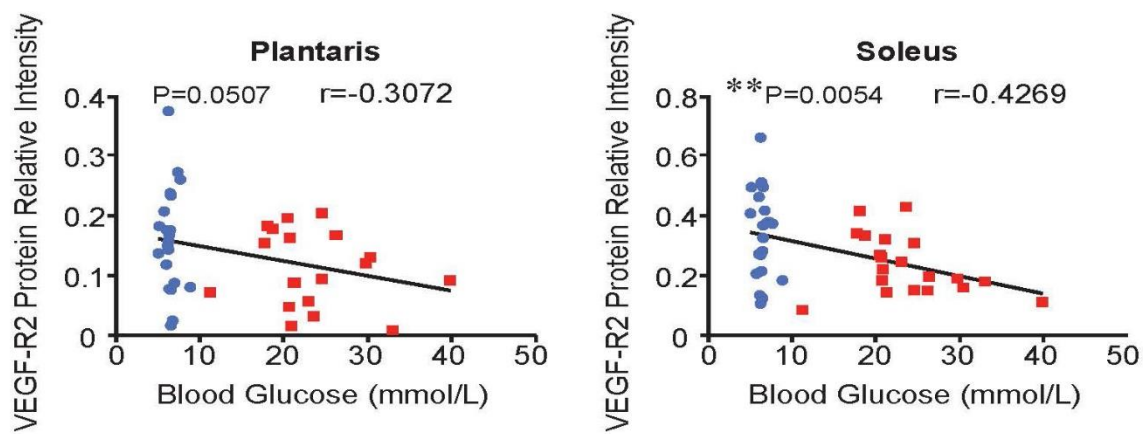
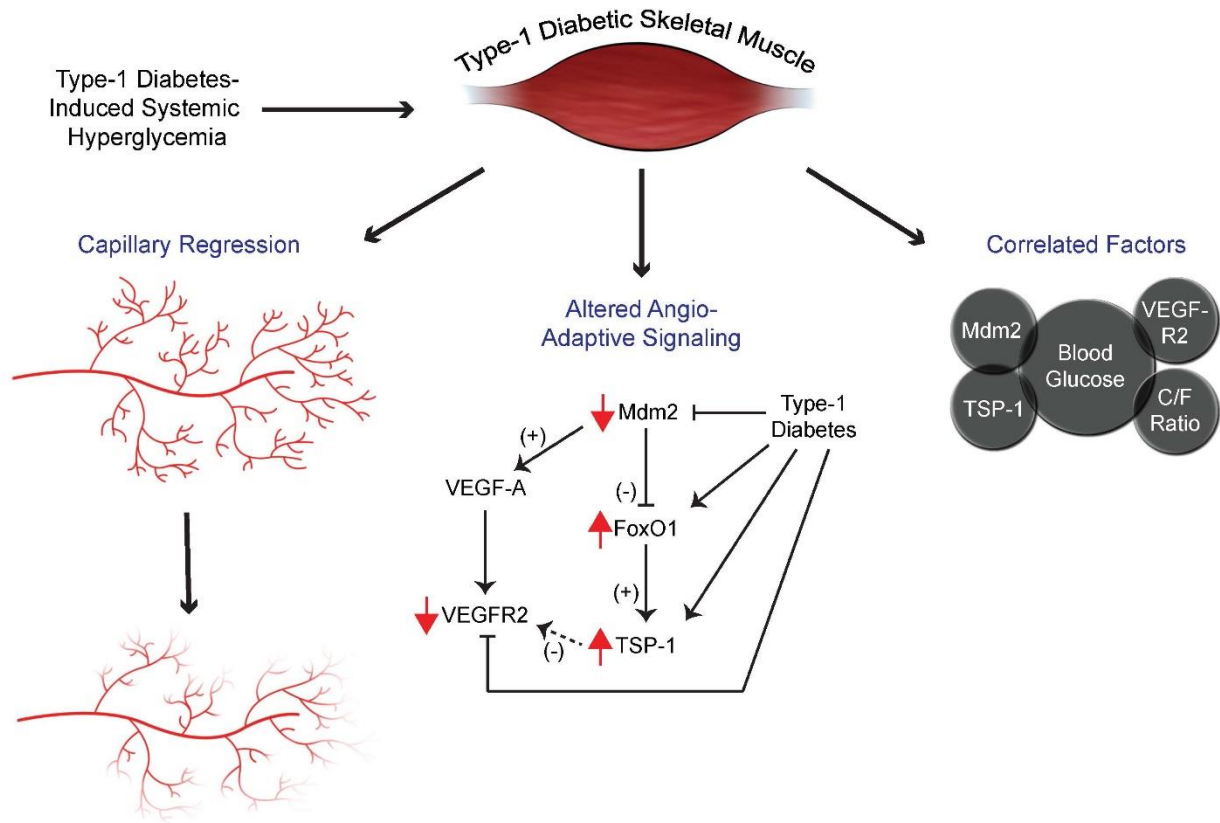
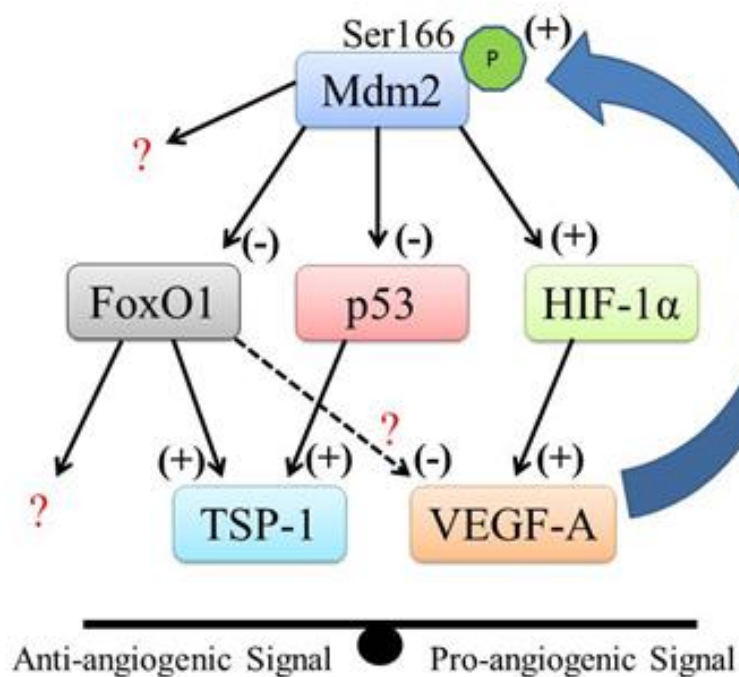


Figure 9.6



## 10. DISCUSSION

The role of Mdm2 in the angiogenic process is very complex, suggesting that Mdm2 may represent a novel ‘master regulator’ of several transcription factors influencing the angio-adaptive balance (FoxO1, HIF-1 $\alpha$ , p53). These interactions affect both pro- and anti-angiogenic factors (VEGF-A, TSP-1), and the interplay between Mdm2 targets further highlights the complexity of the Mdm2-dependent angiogenic process. Refer to figure 10.1 for a schematic representing the various identified interactions between Mdm2 and its downstream targets.



**Figure 10.1** Schematic diagram of the complex regulatory functions of Mdm2 in the skeletal muscle and endothelial cell angio-adaptive balance including the reciprocal relationship between Mdm2 and VEGF-A, (+) indicates stimulatory and (-) indicates inhibitory effects. The letter “P” in a green circle represents a phosphorylated protein.

It has been the scope of my dissertation research to ascertain the physiological role of Mdm2 in regulating the skeletal muscle microvasculature. Ongoing clinical trials investigating drugs that block the interaction between Mdm2 and p53 in order to restore p53 function in cancer cells is intriguing. Although Mdm2 is overexpressed in some disease states (i.e. cancer), its basal expression level appears to be important in other contexts (i.e. in order to prevent muscle capillary regression in type 1 diabetes) and is essential for other healthy and physiological processes (the growth of muscle capillaries in response to exercise). My research highlights the necessity of understanding the role of Mdm2 in healthy tissues such as the skeletal muscle, as Mdm2-centered anti-cancer treatments could have devastating effects to the microvasculature in this tissue. It is of great importance to understand both pathological and physiological functions of Mdm2 in order to optimize existing pharmaceutical interventions and prevent unwanted deleterious effects in non-targeted tissues.

My research took the unique approach of investigating the pro-angiogenic function of Mdm2 in skeletal muscle and in endothelial cells at 4 distinct levels; 1) identifying signalling pathways and mechanisms of Mdm2 activation *upstream of Mdm2*, 2) investigating what happens to *Mdm2 itself* (i.e. protein abundance and post-translational modifications) in response to exercise training and an acute bout of exercise, 3) elucidating the pro-angiogenic effect *downstream of Mdm2* in regard to molecular targets and the functional implications in endothelial cells, and 4) investigating the *clinical relevance* of the Mdm2 protein in the context of type 1 diabetes. Upstream of Mdm2, we have identified VEGF-A as a potent regulator of p-Ser166-Mdm2, uncovering a key pro-angiogenic relationship between Mdm2 and VEGF-A in both primary human endothelial cells and in human skeletal muscle tissue as a whole. Mediating this VEGF-A dependent Mdm2 activation is the ERK1/2-p90RSK signalling cascade, which was found to be

indispensable for VEGF-A-driven Mdm2 phosphorylation in human endothelial cells. Both acute bouts and the accumulation of exercise bouts (training) led to alterations in the activity or abundance of Mdm2 in rodent and human skeletal muscle tissue. We have shown that concomitant with elevated VEGF-A protein expression, Mdm2 is phosphorylated on its serine 166 in response to an acute bout of exercise. In addition, Mdm2, which is predominantly studied as an oncoprotein, is significantly upregulated in response to the physiological stimulus of exercise training in human skeletal muscle, translating findings from our laboratory previously obtained in rodents. Downstream of Mdm2 activation, my research demonstrates that Ser166 phosphorylation on Mdm2 enhances endothelial cell migration and promotes Mdm2/FoxO1 binding and suppressed TSP-1 expression, uncovering a novel pro-angiogenic signalling pathway in endothelial cells. Finally, we have identified Mdm2 as a potentially important regulator of the skeletal muscle microvasculature in the context of diabetes, as Mdm2 protein levels are lowered corresponding to elevations in FoxO1 and TSP-1 alongside significant capillary regression in type 1 diabetic animals.

Throughout my research, I have consistently shown that Mdm2 may exert its pro-angiogenic effects by modulating the Mdm2-FoxO1-TSP-1 signaling axis. While we and others have described Mdm2 as a key regulator of VEGF-A expression [75,147-149], taken together, my research strongly suggests that the anti-angiogenic side of the regulatory balance may be more influential in exerting Mdm2 downstream pro-angiogenic effects. In “*academic research paper 2*” I show that while VEGF-A strongly stimulated the phosphorylation of Mdm2, thereby promoting Mdm2 activity, it was an increased interaction with FoxO1 and subsequent downregulation of FoxO1 transcriptional activity toward hallmark target genes, namely TSP-1, that led to enhanced endothelial cell migration. Interestingly, in cells that overexpressed the phospho-mimetic form of

Mdm2 (S166D-Mdm2), TSP-1 mRNA levels were significantly reduced along with suppressed VEGF-A expression. Despite the reduction in VEGF-A, the VEGF-A/TSP-1 ratio was much greater in cells overexpressing S166D-Mdm2 due to the larger relative reductions in TSP-1. While VEGF-A appears to be an essential driving force behind Mdm2 activation, signaling downstream of Mdm2 appears to be the limiting functional step in the pathway, as the Mdm2 antagonist nutlin-3a entirely prevented the VEGF-A induced cell migration. The Mdm2-FoxO1-TSP-1 axis appears to be this functional link. Similarly, results observed in “*academic research paper 4*” show that in the context of type 1 diabetes, TSP-1 protein levels are significantly elevated concomitant with decreased Mdm2 abundance and capillary regression in the muscle of diabetic rats. VEGF-A protein however were either unaltered (soleus) or significantly higher (plantaris) in diabetic muscle despite the observed loss in capillaries. In both the soleus and plantaris, the VEGF-A/TSP-1 ratio was a better reflection of capillary loss, as this ratio was dramatically reduced in diabetic animals, again due to the relative alterations in TSP-1. Overall, the available literature indeed suggests that skeletal muscle capillary regression is more correlated with increased TSP-1 levels as opposed to reductions in VEGF-A [64,72]. Taken together, my research contributes novel evidence supporting the important role of anti-angiogenic factors in the regulation of the skeletal muscle microvasculature.



## 11. FUTURE RESEARCH

Given the complex nature of Mdm2 interaction with angio-adaptive molecules, it is very likely that other angiogenic regulators are controlled by Mdm2 in skeletal muscle tissue and in endothelial cells. Broadening the scope of potential Mdm2 dependent angiogenic factors could be achieved by performing a Taqman real-time qPCR arrays. These arrays measure the expression level of >40 genes involved in angiogenesis, measured in duplicate. Modulating Mdm2 abundance and activity would allow direct comparison of the expression level of several angiogenic regulators measurable by the array that could be affected by Mdm2 status and activation. To investigate Mdm2 regulation in whole skeletal muscle tissue, arrays could be performed on muscles from Mdm2<sup>Puro/Δ7-9</sup> transgenic mice that are Mdm2 deficient [75]. In primary human endothelial cells, S166D-Mdm2 infected cells could be compared to control and WT-Mdm2 overexpressing cells (see *academic research paper 2*). Changes detected in the array can be confirmed by regular Taqman qPCR (and at the protein level by western blot).

My research significantly contributed to the understanding of both the upstream mechanisms of Mdm2 phosphorylation, and how this activation affects the functionality of *endothelial cells*. Alternatively, the potentially pro-angiogenic role of Mdm2 in the *myofiber* has not yet been identified. It is understood that the skeletal muscle is an endocrine organ [204], and myofibers can be considered the ‘power plant’ behind the production and release of several angiogenic factors that are essential for the health of the capillary network, such as VEGF-A. Indeed, the impact of myofiber-derived VEGF-A for the maintenance of muscle capillaries and for exercise-induced angiogenesis to occur has been reviewed earlier in this dissertation [93-96]. Furthermore, preliminary data from our laboratory shows that cultured muscle cells express TSP-1 mRNA, and secrete TSP-1 protein into culture media (data not shown), identifying the myofiber

as a source of TSP-1 production and release. We have already demonstrated that Mdm2 has the ability to regulate both VEGF-A and TSP-1 expression at the level of whole skeletal muscle tissue and in endothelial cells, yet have not elucidated this relationship specifically in myocytes. Therefore, an intriguing research project would be to investigate Mdm2 regulation of VEGF-A and TSP-1 production and release from skeletal myocytes by manipulating Mdm2 activity and expression with the Mdm2 antagonist nutlin-3a or transfecting cells with Mdm2 siRNA. In addition, we have identified VEGF-A as an upstream regulator of p-Ser166-Mdm2 in endothelial cells, however it is not yet known the upstream stimuli responsible for Mdm2 phosphorylation in skeletal muscle cells.

As outlined in the literature review section, exercise represents a complex environmental stimulus, in which multiple factors could play a role in the observed increase in p-Ser166-Mdm2 protein levels. Mechanical forces such as shear stress and tissue stretch along with localized hypoxia could all serve as pro-angiogenic signals leading to alterations in the angio-adaptive balance, and have the ability to stimulate signaling pathways involved in the promotion of p-Ser166-Mdm2. Indeed, my research clearly shows that Mdm2 acutely senses exercise stimulus, as can be appreciated by the increased phosphorylation of Mdm2 in response to an acute bout of exercise in both rodent and human skeletal muscle tissue (*academic research paper 2*). By utilizing cell culture models, we can mimic and isolate the effect of each exercise-related stressor by exposing cultured endothelial cells and/or myocytes to hypoxic incubation, hydrogen peroxide (oxidative stress), static stretch (Flexcell strain unit), or shear stress (parallel plate flow chambers). Following each respective treatment, p-Ser166-Mdm2 levels could be measured to evaluate which specific exercise-induced stress could be most important for regulating Mdm2 activity. Furthermore, and in combination with potential future research outlined in the previous paragraph,

VEGF-A and TSP-1 production and secretion from myocytes in response to the various stimuli could be measured. The role of Mdm2 in regulating VEGF-A and TSP-1 production could be appreciated by pre-treating cells with nutlin-3a or transfection of Mdm2 siRNA prior to exposure of the exercise-related stressor.

The well-established Mdm2 downstream targets p53, HIF-1 $\alpha$ , and FoxO1 have all been implicated in the regulation of mitochondrial content in various cell types (i.e. cancer cells, skeletal myocytes, and endothelial cells). Namely, it has been shown that p53 plays an important role in the exercise-induced activation of mitochondrial synthesis in skeletal muscle tissue [205], and that an acute bout of exercise significantly induces translocation of the p53 protein to the mitochondria [206]. HIF-1 $\alpha$ , conversely, appears to suppress mitochondrial biogenesis, as prolonged exposure to hypoxia (an event which stabilizes HIF-1 $\alpha$ ) leads to decreased mitochondrial content in skeletal muscle fibres [207]. Furthermore, Mason and Johnson [208] found that while wild-type mice had an increased oxidative capacity, muscle-specific HIF-1 $\alpha$  knockout mice had already developed this adaptation without undergoing an exercise training program. Finally, FoxO1 may also play an important role in the metabolic regulation of endothelial cells, as a recent study by Wilhelm et al. [209] shows that forced expression of FoxO1 in endothelial cells reduces glycolysis and mitochondrial respiration. Using an *in vivo* model of Mdm2 deficiency [75], our laboratory has shown that Mdm2<sup>Puro/ $\Delta$ 7-9</sup> mice who express 60% less Mdm2 than wild-types have an elevated HIF-1 $\alpha$  protein level in their skeletal muscle. These mice display a 20% reduction in skeletal muscle vascularization at rest, potentially leading to a hypoxic environment and HIF-1 $\alpha$  upregulation. Furthermore, the Mdm2 deficient mice had significantly elevated FoxO1 protein levels and expression of the FoxO1 transcriptional target TSP-1. These elevations in HIF-1 $\alpha$  and FoxO1 could be a direct result of Mdm2 deficiency, and may potentially be a contributing factor to

lowered mitochondrial content. To date, it is not known whether Mdm2 could regulate mitochondrial biogenesis and respiration through interactions with its downstream targets. As previously stated, results from our laboratory show that increasing activation of Mdm2 in endothelial cells leads to an elevated binding between Mdm2 and FoxO1, and not between Mdm2 and p53 or HIF-1 $\alpha$  (*academic research paper 2*). The role of Mdm2 in skeletal muscle mitochondrial content and respiration *in vivo* could be assessed in Mdm2<sup>Puro/ $\Delta$ 7-9</sup> mice, while the role of Mdm2 in endothelial and myocyte mitochondrial regulation *in vitro* could be investigated by treating primary human endothelial cells and myotubes with increasing concentrations of nutlin-3a or by transfection with Mdm2 siRNA. Whether the Mdm2-FoxO1 relationship could lead to increases in mitochondrial respiration in endothelial cells would be a novel finding in the field of Mdm2 research.

## 12. CLOSING STATEMENT

It is common knowledge that exercise leads to numerous health benefits, and that by exercising we can become more “in shape” or “fit”. One very important determinant of this end-result is adaptation of the skeletal muscle tissue. However, the muscle tissue would simply not be able to function properly without tightly orchestrated alterations to its vasculature. It has been my passion as a researcher to elucidate the molecular mechanisms that govern this adaptation to exercise. Just prior to the beginning of my graduate studies, the pioneering study from our laboratory that identified Mdm2 as an essential regulator of skeletal muscle capillarization had been published [75]. This research article truly paved the way and plotted the course for my research endeavours.

Our laboratory is one of the few investigating the physiological function of Mdm2. When I began graduate studies in 2012, very little research existed examining the role of Mdm2 outside the context of cancer therapy. While investigating Mdm2 function in cancer is a promising field of research, it has been a great experience being able to contribute to the accumulation of evidence for a physiological role of Mdm2 in healthy tissues such as the skeletal muscle. At the time of writing this dissertation, our laboratory has solidified Mdm2 as an essential regulator of the muscle microvasculature through several research publications and scientific collaborations. I have dissected the pro-angiogenic role of Mdm2 in response to exercise training, as well as in response to individual acute bouts of exercise. Our laboratory has implemented an integrative approach to the study of Mdm2 angio-regulation, and I have contributed to the identification of novel interactions and signalling pathways involving Mdm2 and other angio-adaptive molecules in rodent and human skeletal muscle and in primary human endothelial cells. Additionally, my research has generated findings that may contribute in the long term to improving the treatment of

diseases such as type 1 diabetes that affect the muscle vasculature. Taken together, my doctoral research provides an important contribution to the field of muscle biology and exercise physiology, and improves our understanding of the molecular mechanisms regulating microvascular adaptability of skeletal muscle to physical exercise. It has been a privilege to immerse myself in the detailed investigation of skeletal muscle capillary plasticity in health and disease.

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## 14. APPENDIX A: ACADMIC RESEARCH PAPER 5

### **The effects of voluntary exercise and prazosin on capillary rarefaction and 2 metabolism in streptozotocin-induced diabetic male rats**

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#### **Author Contributions:**

Julian Aiken:

- Performed western blotting experiments to measure Mdm2, VEGF-A, and TSP-1 in both tibialis anterior and soleus muscles
- Participated in data analysis
- Participated in manuscript preparation including figure preparation for western blotting (Mdm2, VEGF-A, TSP-1)



**The effects of voluntary exercise and prazosin on capillary rarefaction and metabolism in streptozotocin-induced diabetic male rats**

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Running head: Exercise and Prazosin Cooperatively Augment Angiogenesis in T1D

## Abstract

Type-1 diabetes mellitus (T1D) causes impairments within the skeletal muscle microvasculature. Both regular exercise and prazosin have been shown to improve skeletal muscle capillarization and metabolism in healthy rats through distinct angiogenic mechanisms. The aim of this study was to evaluate the independent and additive effects of voluntary exercise and prazosin treatment on capillary-to-fiber ratio (C:F) in streptozotocin (STZ)-treated diabetic rats. STZ (65 mg/kg) was intraperitoneally administered to male Sprague-Dawley rats ( $n = 36$ ) to induce diabetes, with healthy, nondiabetic, sedentary rats ( $n = 10$ ) as controls. The STZ-treated rats were then divided into sedentary (SED) or exercising (EX; 24-h access to running wheels) groups and then further subdivided into prazosin (Praz) or water (H<sub>2</sub>O) treatment groups: nondiabetic-SED-H<sub>2</sub>O, STZ-SED-H<sub>2</sub>O, STZ-EX-H<sub>2</sub>O, STZ-SED-Praz, and STZ-EX-Praz. After 3 wk, untreated diabetes significantly reduced the C:F in tibialis anterior (TA) and soleus muscles in the STZ-SED-H<sub>2</sub>O animals (both  $P < 0.05$ ). Voluntary exercise and prazosin treatment independently resulted in a normalization of C:F within the TA ( $1.86 \pm 0.12$  and  $2.04 \pm 0.03$  vs  $1.71 \pm 0.09$ ,  $P < 0.05$ ) and the soleus ( $2.36 \pm 0.07$  and  $2.68 \pm 0.14$  vs  $2.13 \pm 0.12$ ,  $P < 0.05$ ). The combined STZ-EX-Praz group resulted in the highest C:F within the TA ( $2.26 \pm 0.07$ ,  $P < 0.05$ ). Voluntary exercise volume was negatively correlated with fed blood glucose levels ( $r^2 = -0.7015$ ,  $P < 0.01$ ) and, when combined with prazosin, caused further enhanced nonfasted glucose ( $P < 0.01$ ). Exercise and prazosin reduced circulating nonesterified fatty acids more than either stimulus alone ( $P < 0.05$ ). These results suggest that the distinct stimulation of angiogenesis, with both regular exercise and prazosin treatment, causes a cooperative improvement in the microvascular complications associated with T1D.

## **Introduction**

Type-1 diabetes mellitus (T1D) is a chronic autoimmune disease targeting the pancreatic  $\beta$ -cells resulting in little to no insulin production and hyperglycemia (8). Despite exogenous insulin therapy, individuals with diabetes have an increased risk for long-term microvascular and macrovascular complications, which can significantly impact their morbidity and mortality (7, 10). T1D is also associated with impaired angiogenesis in skeletal muscle (1, 38). It is generally held that hyperglycemia itself, or some metabolic byproduct of hyperglycemia, induces remodeling of capillaries within the skeletal muscle, resulting in a lower capillary-to-fiber ratio (C:F) and ultimately affecting regional hemodynamic regulation (26, 41).

New capillary growth within the skeletal muscle occurs via existing capillaries through two morphologically different, and separately inducible, forms of physiological angiogenesis termed sprouting or nonsprouting angiogenesis (13). The growth of new capillaries in response to exercising muscle is a highly regulated process (16), which is stimulated by increased functional hyperemia and shear stress (19), mechanical stretch of the tissue in addition to increases in metabolic demand, or reduced oxygen delivery (12). These signals modulate the expression and activity of proangiogenic and angiostatic factors, which act together to regulate sprouting angiogenesis (13).

In addition to insulin therapy, regular exercise is an established management strategy for T1D, improving glucose uptake and insulin sensitivity within skeletal muscle (15). Additional health benefits include increased cardiorespiratory fitness, improved endothelial function, increased vascular health, and quality of life (6, 15). Regular endurance exercise increases skeletal muscle capillarization in healthy individuals (22). However, the effects of endurance training on changes in capillarization in diabetic animals and patients are contradictory (20, 27, 29, 44).

Vascular function has been shown to improve with physical activity in both animal models of diabetes (27) and in patients with T1D (6), but these alterations were unable to fully restore the diabetes-induced defects (14, 33).

Prazosin, an  $\alpha$ 1-adrenergic antagonist, increases skeletal muscle capillarization via a vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase-dependent pathway (eNOS) (5, 45). These two mechanisms of action for prazosin ultimately cause longitudinal splitting of existing capillaries (46), which is distinct from the angiogenic, or sprouting, mechanisms that are observed with exercise. Prazosin has been used in many rodent studies (3, 9, 47) to selectively increase skeletal muscle C:F, but it has yet to be used as an agent to stimulate angiogenesis in an animal model of T1D. Chronic treatment with prazosin in streptozotocin (STZ)-induced diabetic rats reduces elevations in blood pressure and cholesterol levels and improves cardiac function (18), but its effect in muscle is unknown in the STZ model. In humans with type 2 diabetes, prazosin enhances hepatic function (11, 34), improves insulin sensitivity, and lowers lipid levels (21, 32), in addition to decreasing blood pressure (11, 21, 32, 34).

The aim of this study was to evaluate the independent and combined effects of exercise and prazosin administration on skeletal muscle capillary content in the STZ-induced rodent model of T1D.

## **Methods**

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and was approved by the York University Animal Care Committee (2013–5). The *Guide for the Care and Use of Laboratory Animals* (8th ed., 2011) was followed.

*Animals.* Adult, male Sprague-Dawley rats (Charles River Laboratories; initial mass of 225–250 g,  $n = 46$ ) were individually housed (lights on 12-h cycle: lights off 12-h cycle) after 1 wk of acclimatization to room temperature (22–23°C)- and humidity (50–60%)-controlled facilities.

*Experimental design.* A timeline of the experimental protocol is shown in Fig. 14.1. All animals had access to voluntary running wheels for 1 wk. Each exercising animal was placed into rodent cages with 24-h access to a running wheel (Harvard Apparatus), while sedentary animals were housed in standard cages. Running distance was assessed daily. Seven days after wheel assignment, a single injection of STZ (65 mg/kg) was administered to induce diabetes. Animals were also provided with sugar water (20% sucrose) to assist with diabetes development, and any exercising animals had their wheels removed overnight to further promote diabetes induction. Two days after diabetes inducement, animals were divided into one of five groups: nondiabetic-SED-H<sub>2</sub>O (control), STZ-SED-H<sub>2</sub>O, and STZ-EX-H<sub>2</sub>O, all given regular drinking water; and STZ-SED-Praz and STZ-EX-Praz, all given drinking water containing prazosin hydrochloride (5 mg/kg; P7791; Sigma-Aldrich). Five days after diabetes induction, all animals had their food reduced to 30% of their total body weight to improve glycemia in the diabetic animals. Body mass, fed blood glucose, food intake, and fluid consumption were measured daily for each rodent and any changes in the rodents' health were noted and monitored.

*Blood glucose, nonesterified fatty acid, and corticosterone sampling.* Blood glucose values were measured with a glucometer (Alpha-TRAK; Abbott Laboratories) and 5  $\mu$ l of blood from the tip of the tail. Blood samples were collected from each animal via saphenous vein for nonesterified fatty acid (NEFA kit, HR Series NEFA-HR; Wako Chemicals) and corticosterone (MP Biomedical) concentration measurements on *days 1* (basal) and *32*. Glucose area under the curve

(AUC) was measured relative to each animal's individual *day 11* glucose value and the net area was used to account for the lowering in blood glucose observed in both exercising groups.

*Capillary-to fiber analysis.* Skeletal muscle, tibialis anterior (TA), and soleus, from euthanized animals, was embedded in tissue freezing medium, frozen in liquid nitrogen, and cryosectioned (10- $\mu$ m thick). TA and soleus sections (10- $\mu$ m thick) were fixed with 3.7% paraformaldehyde before being stained with fluorescein isothiocyanate-conjugated Griffonia simplicifolia isolectin B4 (1:100; Vector Laboratories). Sections were viewed using a Zeiss M200 inverted microscope with a X20 objective and images were captured using Metamorph imaging software. Capillary-to-fiber counts were averaged from five to seven independent fields of view per animal by a blinded observer, and ~45 fibers/image were counted.

*Western blotting.* Immunoblotting was carried out on protein extracts from rat soleus or TA muscles as previously described (2). Frozen muscle (20-40 mg) was mixed at 4°C with RIPA buffer. For each sample, protein extracts were prepared using two stainless carbide beads (Retsch, Fisher Scientific, Montreal, Canada) in the Retsch MM400 tissue lyser (30 pulses/s; Retsch, Haan, Germany). Denatured samples (30  $\mu$ g/well) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. After blocking with 5% fat free milk at room temperature for 45 min, the blots were probed overnight at 4°C with primary antibodies against the following proteins:  $\beta$ -actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA), murine double minute-2 (Mdm2; noncommercial clone 2A10 (2, 39, 40), VEGF (clone VG-1; 05-1117; Millipore, Etobicoke, ON, Canada), or TSP-1 (clone A6.1; MS-421-P0; Invitrogen, Burlington, ON, Canada). After incubation with secondary antibody (cat. no. P0260; Dako, Carpinteria, CA), proteins were visualized with chemiluminescence (Millipore) on Imaging Station 4000MM Pro (Carestream

Health, Rochester, NY) or on X-ray film (CL-XPosure Film; prod. no. 34090; Thermo Scientific, Rockford, IL). Blots were analyzed with Carestream software.

*Statistical analysis.* All data are represented as means  $\pm$  SE, with a criterion of  $P < 0.05$  and  $P < 0.01$  and were assessed as stated using two-way ANOVAs as a means of statistical significance. Individual differences were calculated using Bonferroni's post hoc test (Statistica, StatSoft). A *t*-test was also used to compare values for nondiabetic and STZ-treated rats.

## Results

*Capillary-to-fiber content.* The effect of STZ-induced diabetes on the skeletal muscle microvasculature was evaluated using the C:F, expressed relative to fiber number to account for diabetes-related muscle atrophy. There was obvious capillary rarefaction in the TA of the STZ-SED-H<sub>2</sub>O group when compared with the nondiabetic, control group (Fig. 14.2, A and A',  $P < 0.001$ ), which was paralleled within the soleus of the STZ-SED-H<sub>2</sub>O animals (Fig. 14.3, A and A',  $P < 0.001$ ).

Within the TA, C:F for both the STZ-SED-Praz and the STZ-EX-H<sub>2</sub>O groups were significantly higher from the STZ-SED-H<sub>2</sub>O group (Fig. 14.2, B and B',  $P < 0.001$ ), although they were not significantly different from each other. This suggests that individually, both prazosin and exercise were capable of improving the diabetes-induced loss of capillaries toward that observed in nondiabetic control rats. The STZ-EX-Praz animals had significantly higher C:F in comparison with the STZ-EX-H<sub>2</sub>O and STZ-SED-Praz animals ( $P < 0.001$ ), signifying that cotreatment exerted an additive effect on angiogenesis. We also observed an increase in C:F within the soleus of the STZ-SED-Praz animals ( $P < 0.01$ ) and the STZ-EX-Praz animals (Fig. 14.3, B and B',  $P < 0.05$ ); however, exercise was unable to improve the diabetes-induced rarefaction independently.

*Circulating glucose concentrations.* Hyperglycemia was evident in all STZ-treated animals (Fig. 14.4A). When analyzed as an AUC from *day 11* onward (the date of prazosin initiation), there was a significant main effect of both exercise and prazosin to improve daily fed glucose concentrations, with the most favorable response occurring in the STZ-EX-Praz group (Fig. 14.4A',  $P < 0.01$ ), suggesting that the cotreatment produced the most beneficial result.

*Running distance.* Daily running distance was graphed in relation to prazosin administration (Fig. 14.5A) and no difference was found between the average running distance in the two exercising groups (Fig. 14.5B). A significant negative correlation was observed between individual mean blood glucose concentrations and cumulative running distances ( $r^2 = -0.70$ ; Fig. 14.5C,  $P < 0.01$ ).

*Corticosterone and NEFAs.* STZ-induced diabetes affected the concentrations of circulating corticosterone and NEFAs. Before prazosin administration, corticosterone and NEFA concentrations were not different across the STZ-treatment group, so these data were pooled (initial basal, Fig. 14.6, A and B). All STZ-treated animals developed significantly elevated corticosterone concentrations (Fig. 14.6A,  $P < 0.001$ ) at the end of the treatment period, and only the prazosin group showed significantly decreased values (Fig. 14.6A',  $P < 0.05$ ). There was a significant effect of voluntary exercise, prazosin and their combination, to improve NEFA values (Fig. 14.6B',  $P < 0.05$ ).

*Mdm2, VEGF-A, and TSP-1.* VEGF-A and TSP-1, key pro- and antiangiogenic molecules in skeletal muscle, are influenced by Mdm2, and these three markers have been used to understand the changes observed in skeletal muscle C:F. In both the TA and soleus muscles, STZ-treated rats had significantly decreased Mdm2 protein content vs. nondiabetic rats (Fig. 14.7, A, A', B, and B',  $P < 0.05$ ). Within the TA, Mdm2 protein content was lowest in the STZ-SED-H2O group but was



only significantly lower than the STZ-SED-Praz group (Fig. 14.7A",  $P > 0.05$ ). Soleus Mdm2 protein content was not significantly modified in the four different treatment groups (Fig. 14.7B"). There was no significant effect of STZ treatment, prazosin, or exercise on VEGF-A protein content within either the TA or soleus muscles (Fig. 14.8, A, A', A", B, B', and B",  $P > 0.05$ ). There was also no significant effect of STZ treatment, prazosin, or exercise on TSP-1 protein content within either the TA or soleus muscles (Fig. 14.9, A, A', A", B, B', and B",  $P > 0.05$ ).

## **Discussion**

Using a combined therapeutic approach, we have shown that exposure to 19 days of combined voluntary exercise and prazosin treatment caused improved skeletal muscle vascularization, through enhanced C:F within the TA muscle and a normalization of the C:F within the soleus muscle of STZ-treated rats. Acutely, prazosin administration is not known to improve glycemia in diabetes; however, we found that prazosin-induced skeletal muscle capillarization, which was independent of voluntary exercise, was associated with improvements in nonfasted (fed) glucose concentrations. When combined with voluntary exercise, prazosin treatment caused additional improvements in fed glucose concentrations and circulating lipid levels, thereby suggesting that the two treatments can act in an additive fashion to improve muscle capillarization and metabolism in this animal model of T1D. To our knowledge, this is the first time that these two therapeutic modalities have been combined in an animal model (health or disease) and suggests that there is likely an additive effect of both prazosin and exercise on angiogenesis and metabolic rescue in diabetes.

It is well established that endurance exercise promotes new capillary growth (22) in both animals and healthy humans (35); however, literature regarding the effect of exercise on the pathological effects of T1D within the skeletal muscle vasculature is inconclusive (20, 44). In

healthy rats, exercise training causes increased VEGF mRNA and protein content, which stimulates further enhancements in muscle capillarization (16). When examined in STZ-induced diabetic mice, treadmill endurance training was unable to improve quadriceps femoris cross-sectional fiber area or C:F after 5 wk of exercise training (27). In our study, uncontrolled STZ-induced diabetes caused significant capillary rarefaction, a decrease in the number of capillaries in an area of tissue, in both the TA (Fig. 14.2, *A* and *A'*) and soleus muscles (Fig. 14.3, *A* and *A'*), which is consistent with previous studies (28, 41). When given access to voluntary exercise wheels, STZ-induced diabetic rats showed a significant increase in C:F in the TA (Fig. 14.2, *B* and *B'*) and a slight, but not significant, tendency to increase C:F in the soleus (Fig. 14.3, *B* and *B'*) when compared with sedentary STZ-treated rats. The exercise modality in our study was voluntary wheel running and this might have elicited different muscle recruitment between the TA vs. soleus muscles (25), although there was no difference in volume of exercise between treatment groups (Fig. 14.5, *A* and *B*). Additionally, angiogenic potential appears to be inversely proportional to the original capillarity, i.e., it is easier to induce in fast muscle than slow or cardiac muscle (12), another possible explanation for the muscle type-specific differences observed in capillarity.

The enhancement of skeletal muscle capillarization, through nonsprouting angiogenesis with prazosin treatment, results in an increase in capillarity in glycolytic and oxidative skeletal muscles in healthy rats (3, 9, 47). However, to our knowledge, this study is the first to illustrate the beneficial effects of prazosin on skeletal muscle capillary rarefaction in STZ-induced diabetic rats. We observed a normalization of C:F in both TA and soleus muscles of sedentary, STZ-treated rats as their C:F values were equivalent to those measured in the control group. When coupled with wheel running, there was an additive effect on the angiogenic response, specifically in the TA. It is worth noting that the skeletal muscle angiogenesis observed within both the TA and

soleus (the normalization) occurred amidst significantly elevated circulating corticosterone levels (Fig. 14.6, A and A'), a hormone that has been linked to capillary rarefaction (42). In addition to the fact that both treatment modalities result in distinct forms of angiogenesis, there are data suggesting that the combination of high-volume, low-intensity exercise, like voluntary wheel running, actually elicits a more rapid angioadaptive response than what is observed after treadmill exercise training (35).

Exercise is a widely recognized strategy for improving glycemia in diabetes (6), as regular exercise leads to the improvement of whole body glucose and lipid metabolism and enhanced skeletal muscle glucose disposal (17) and insulin sensitivity (23, 24). We observed a significant effect of voluntary exercise to improve fed glucose concentrations in the STZ-treated rats, and this result was amplified when the animals were coadministered prazosin (Fig. 14.4, A and A'). These improvements in blood glucose, regardless of prazosin administration, were also correlated to running distance (Fig. 14.5C), suggesting that higher running volumes caused better fed glucose concentrations. In our study, sedentary STZ-induced diabetic rats treated with prazosin also showed improved fed glucose values throughout the experimental protocol, an unexpected but interesting observation, given the inconsistent findings on prazosin treatment in various models of hyperglycemia (4, 18, 31). As prazosin is not known to acutely lower blood glucose levels, our results highlight the importance of skeletal muscle capillary content on glucose homeostasis and demonstrate that increased capillarization likely enhances insulin sensitivity and glucose disposal, an observation recently seen in healthy rats (3).

The angiogenic response is controlled by a dynamic balance between anti- and proangiogenic factors (36). VEGF-A and TSP-1 are key pro- and antiangiogenic molecules (30, 37). Interestingly, we have recently brought evidence that the E3 ubiquitin ligase Mdm2 could be

a central regulator of skeletal muscle angiogenesis, partly by regulating VEGF-A and TSP-1 expression (2, 40).

Our current results show, for the first time, that STZ-treated rats had a significant decrease in Mdm2 protein levels in both the TA and soleus muscles, as summarized in Fig. 14.10A. This reduction in Mdm2 could explain the lowered skeletal muscle capillarization in both muscles in sedentary diabetic rats, as Mdm2 protein levels have been shown to be closely related to endothelial content within the skeletal muscle (40). It was previously observed that both capillarization and Mdm2 protein levels were significantly lower in a model of type-2 diabetes and in skeletal muscles from Zucker diabetic fatty (ZDF) rats (40). Further results demonstrated that voluntary running efficiently restored both the impaired skeletal muscle capillarization and Mdm2 protein levels within the ZDF rats. In contrast to these findings, the voluntary exercise stimulus applied within our current study did not rescue the observed decrease in Mdm2 protein in the diabetic animals (Fig. 14.10B) and suggests that the physiological response of Mdm2 to exercise could be lost in uncontrolled, T1D muscle.

No significant alterations in TSP-1 or VEGF-A protein levels in response to diabetes induction, prazosin treatment, or voluntary exercise were observed in either the TA or soleus muscles. Moreover, VEGF receptor expression in soleus and TA muscle were unchanged by STZ treatment nor by exercise or prazosin treatment (data not shown). Kivelä et al. (27) have shown that STZ-induced diabetic mice had lower VEGF-A protein levels at 3 and 5 wk post-diabetes induction and a concomitant increase in TSP-1 expression. In that study, while exercise served to delay the reduction in VEGF-A levels, it was not sufficient to attenuate the elevation in TSP-1 mRNA. The C:F is increased as early as 14 days of treadmill running in rodent skeletal muscle (43); therefore, we hypothesize that alterations in VEGF-A and TSP-1 protein levels could have

occurred at an earlier time point to stimulate the growth of capillaries. While VEGF-A protein could have been elevated before the measured time point to induce the angiogenic process, the trend for TSP-1 protein levels could indicate the stopping or slowing down of capillary growth, as the balance between oxygen and nutrient supply and demand had been achieved in the skeletal muscle.

In summary, the significant improvement to fed glucose concentration observed after cotreatment with voluntary exercise and prazosin administration could be the result of enhancements in insulin sensitivity and lipid metabolism, increased skeletal muscle glucose disposal, and possibly improved diffusion conditions for glucose in the muscle as the result of heightened skeletal muscle angiogenesis. These results suggest that the combination of both voluntary exercise and prazosin administration could lead to a cooperative improvement in peripheral vascular complications linked to T1D and may perhaps prevent future complications through the augmentation of skeletal muscle capillarization and glycemia status.

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### **Author contributions**

E.C.D., E.L., J.A., and E.R.M. performed experiments; E.C.D., E.L., J.A., E.R.M., and M.C.R. analyzed data; E.C.D., E.L., J.A., E.R.M., T.L.H., O.B., and M.C.R. interpreted results of

experiments; E.C.D., E.L., J.A., and E.R.M. prepared figures; E.C.D., E.L., J.A., E.M., and O.B. drafted manuscript; E.C.D., E.L., J.A., E.R.M., T.L.H., O.B., and M.C.R. edited and revised manuscript; E.C.D., E.L., J.A., E.R.M., T.L.H., O.B., and M.C.R. approved final version of manuscript.

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## Figure legends

Figure 14.1. Schematic of the experimental design. Basal saphenous vein measurement for corticosterone and nonesterified fatty acid (NEFA) concentrations occurred on *day 1* before the provision of voluntary running wheels. Streptozotocin (STZ) was administered on *day 7*, prazosin on *day 11* and on *day 14* animals had their food reduced to 30% of their total body weight to improve glycemia in the diabetic animals. Animals were euthanized on *day 32* (end point) with a final saphenous vein sample. T1D, type 1 diabetes.

Figure 14.2. Capillary-to-fiber ratio (C:F) in the tibialis anterior in nondiabetic and STZ-treated rats (*A* and *A'*) and after 20 days of voluntary exercise and prazosin cotreatment (*B* and *B'*). All values are means  $\pm$  SE ( $n = 3-5$  per group). SED, sedentary; EX, exercise; Praz, prazosin. \*\*\*Significantly different from nondiabetic group ( $P < 0.05$ ). Different letters (a, b, and c) indicate a significant difference (main effect treatment and activity) between groups ( $P < 0.001$ ), following two-way ANOVA (post hoc test).

Figure 14.3. C:F in the soleus of nondiabetic and STZ-treated rats (*A* and *A'*) and after 20 days of voluntary exercise and prazosin cotreatment (*B* and *B'*). All values are means  $\pm$  SE ( $n = 5$  per group). \*\*\*Significantly different from nondiabetic group ( $P < 0.001$ ). Different letters (a, b, and c) indicate a significant difference (main effect of treatment) between groups ( $P < 0.05$ ), using a two-way ANOVA (post hoc test).

Figure 14.4. Blood glucose values across the experimental timeline (*A*) and expressed as area under the curve (AUC; *A'*). AUC values are calculated from *day 11* (prazosin administration) until *day 32* (end point). A main effect of both prazosin ( $P < 0.01$ ) and exercise ( $P < 0.01$ ) to improve daily glucose concentrations was found, with the cotreatment (STZ-EX-Praz) producing the most

significant improvement. All values are means  $\pm$  SE ( $n = 6-10$  per group). Different letters (a, b, and c) indicate a significant difference (main effect treatment and activity) between groups ( $P < 0.05$ ), following two-way ANOVA (post hoc test).

Figure 14.5. Voluntary running distances over time (A) and the total mean values (B) were graphed from the initiation of the cotreatment (prazosin administration). Running distance was negatively correlated to mean blood glucose concentration (C) ( $r^2 = -0.7015$ ,  $P < 0.01$ ). All values are means  $\pm$  SE ( $n = 6-10$ ).

Figure 14.6. Corticosterone and NEFA in nondiabetic and STZ-treated rats (A and B) and after 20 days of voluntary exercise and prazosin cotreatment (A') and (B'). Black and white bar represents combined basal value before STZ injection. All values are means  $\pm$  SE ( $n = 6-10$  per group). \*\*\*Significantly different from nondiabetic group ( $P < 0.001$ ). Different letters (a and b) indicate a significant difference (main effect treatment/activity for corticosterone and activity for NEFA) between groups (all  $P < 0.05$ ), following two-way ANOVA (post hoc test).

Figure 14.7. Skeletal muscle murine double minute-2 (Mdm2) protein levels are decreased in STZ-induced T1D. A: murine double minute-2 (Mdm2) protein in control (nondiabetic) and STZ-treated rat tibialis anterior (TA) muscles. A': Mdm2 protein in nondiabetic and STZ-treated rat TA muscle. \* $P \leq 0.01$  following unpaired Student's *t*-test analysis. A'': Mdm2 protein in the TA muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. Different letters indicate significant difference,  $P \leq 0.05$  following two-way ANOVA. B: Mdm2 protein in nondiabetic and STZ-treated rat soleus muscles. B': Mdm2 protein in nondiabetic and STZ-treated rat soleus muscle. \* $P \leq 0.05$  following unpaired Student's *t*-test analysis. B'': Mdm2 protein in the soleus muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. All data are means  $\pm$  SE ( $n = 6$  per group). Same

letter (a) indicates no significant differences were found between groups following two-way ANOVA.

Figure 14.8. STZ-induced diabetes does not alter skeletal muscle vascular endothelial growth factor (VEGF) protein levels. *A*: VEGF protein in control (nondiabetic) and STZ-treated rat TA muscles. *A'*: VEGF protein in nondiabetic and STZ-treated rat TA muscle. No significant difference following unpaired Student's *t*-test analysis. *A''*: VEGF protein in the TA muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. *B*: VEGF protein in nondiabetic and STZ-treated rat soleus muscles. *B'*: VEGF protein in nondiabetic and STZ-treated rat soleus muscle. No significant difference following unpaired Student's *t*-test analysis. *B''*: VEGF protein in the soleus muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. All data are means  $\pm$  SE ( $n = 6$  per group). Same letters indicate no significant differences between groups following two-way ANOVA.

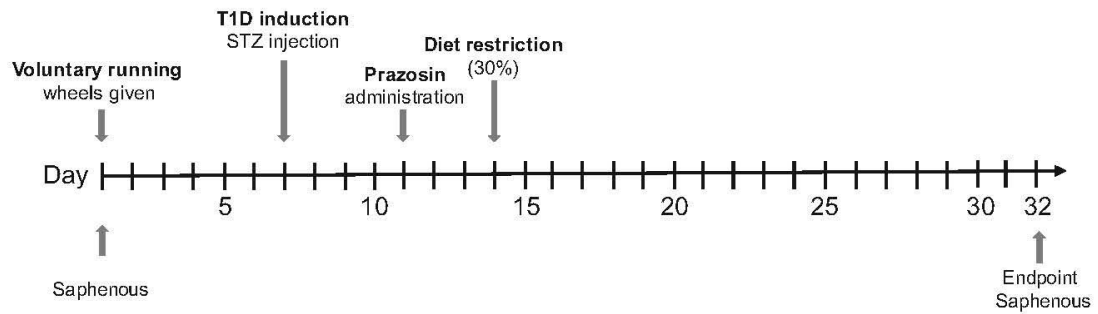
Figure 14.9. STZ-induced diabetes does not alter skeletal muscle thrombospondin-1 (TSP-1) protein levels. *A*: TSP-1 protein in control (nondiabetic) and STZ-treated TA muscles. *A'*: TSP-1 protein in nondiabetic and STZ-treated rat TA muscle. No significant difference following unpaired Student's *t*-test analysis. *A''*: TSP-1 protein in the TA muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. *B*: TSP-1 protein in nondiabetic and STZ-treated rat soleus muscles. *B'*: TSP-1 protein in nondiabetic and STZ-treated rat soleus muscle. No significant difference following unpaired Student's *t*-test analysis. *B''*: TSP-1 protein in the soleus muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. All data are means  $\pm$  SE ( $n = 6$  per group). Same letters indicate no significant differences between groups following two-way ANOVA.



Figure 14.10. Summary of the effects of prazosin and exercise within the TA skeletal muscle of STZ-treated rats. *A*: STZ treatment causes increased fed blood glucose levels, elevations in corticosterone (Cort), NEFA, and decreased insulin (Ins) content. Within the TA, STZ treatment decreased C:F and Mdm2 protein content while both VEGF and TSP-1 were unaffected. *B*: STZ-treated rats were exposed to either prazosin treatment, voluntary exercise, or a combination of both. Individually, prazosin and voluntary exercise increased C:F and decreased both NEFA and fed blood glucose concentrations. Prazosin treatment increased Mdm2 protein content while VEGF and TSP-1 were unaffected. Voluntary exercise caused no alterations to angiogenic proteins. The combination of prazosin with voluntary exercise resulted in the most improved fed blood glucose concentrations and C:F. Both Cort and NEFA concentrations were unaffected and there were no alterations to angiogenic proteins.

Figure 14.1

**Protocol**



**Groups**

Diabetic	STZ	Sedentary	H <sub>2</sub> O	n=6
	STZ	Exercise	Prazosin	n=10
	STZ	Sedentary	Prazosin	n=10
	STZ	Exercise	H <sub>2</sub> O	n=10
Healthy-Control	Sedentary	H <sub>2</sub> O	n=10	

Figure 14.2

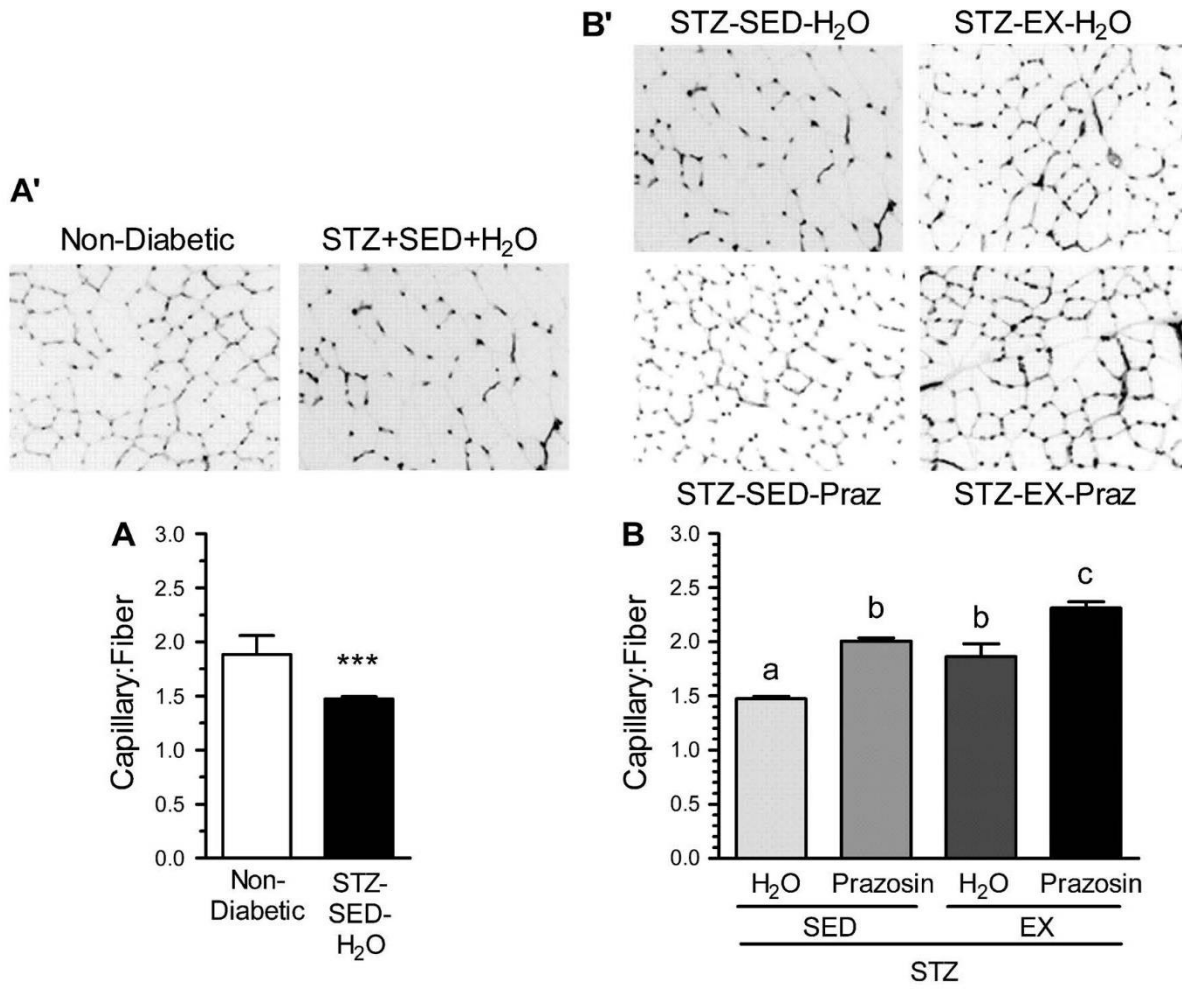


Figure 14.3

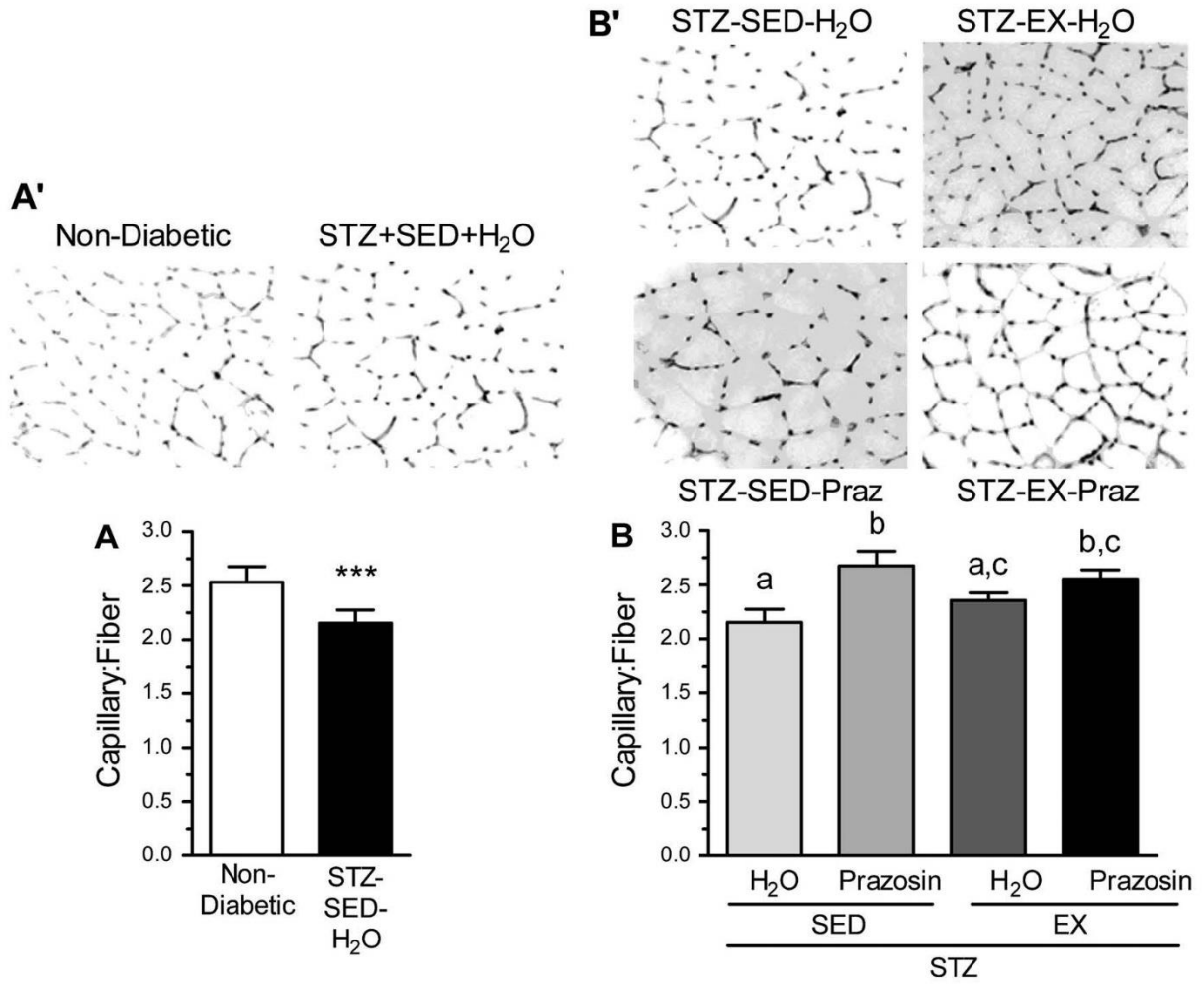


Figure 14.4

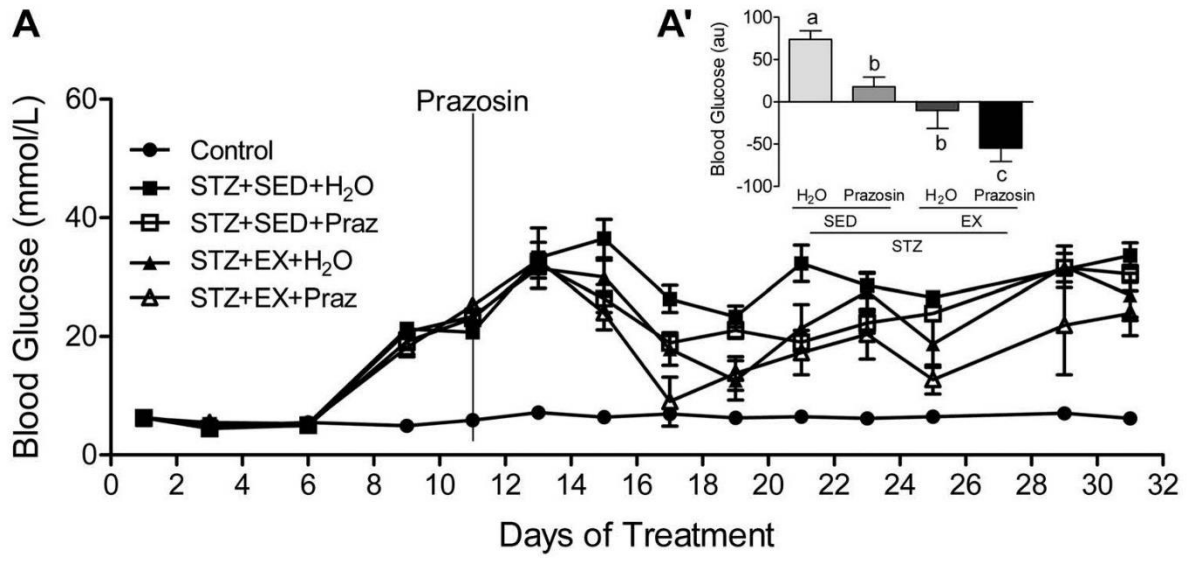


Figure 14.5

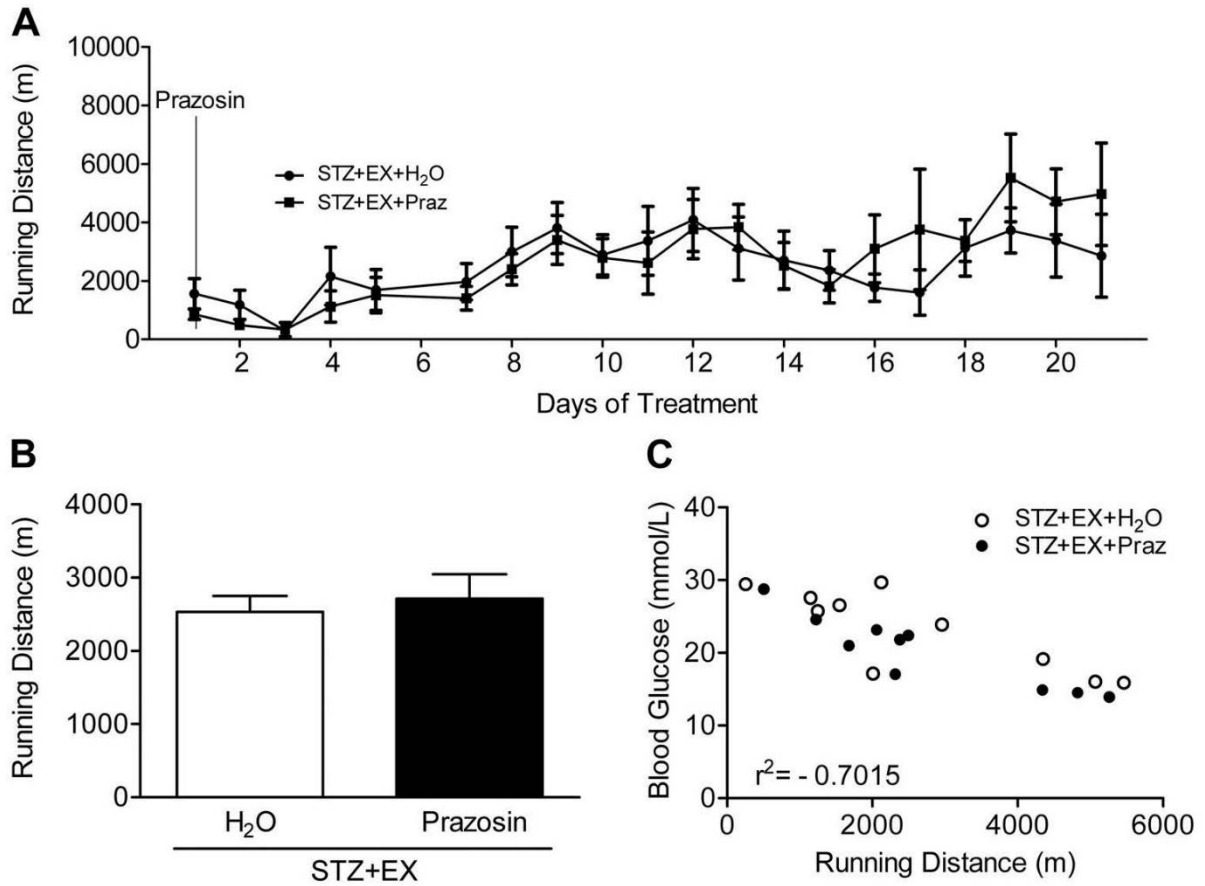
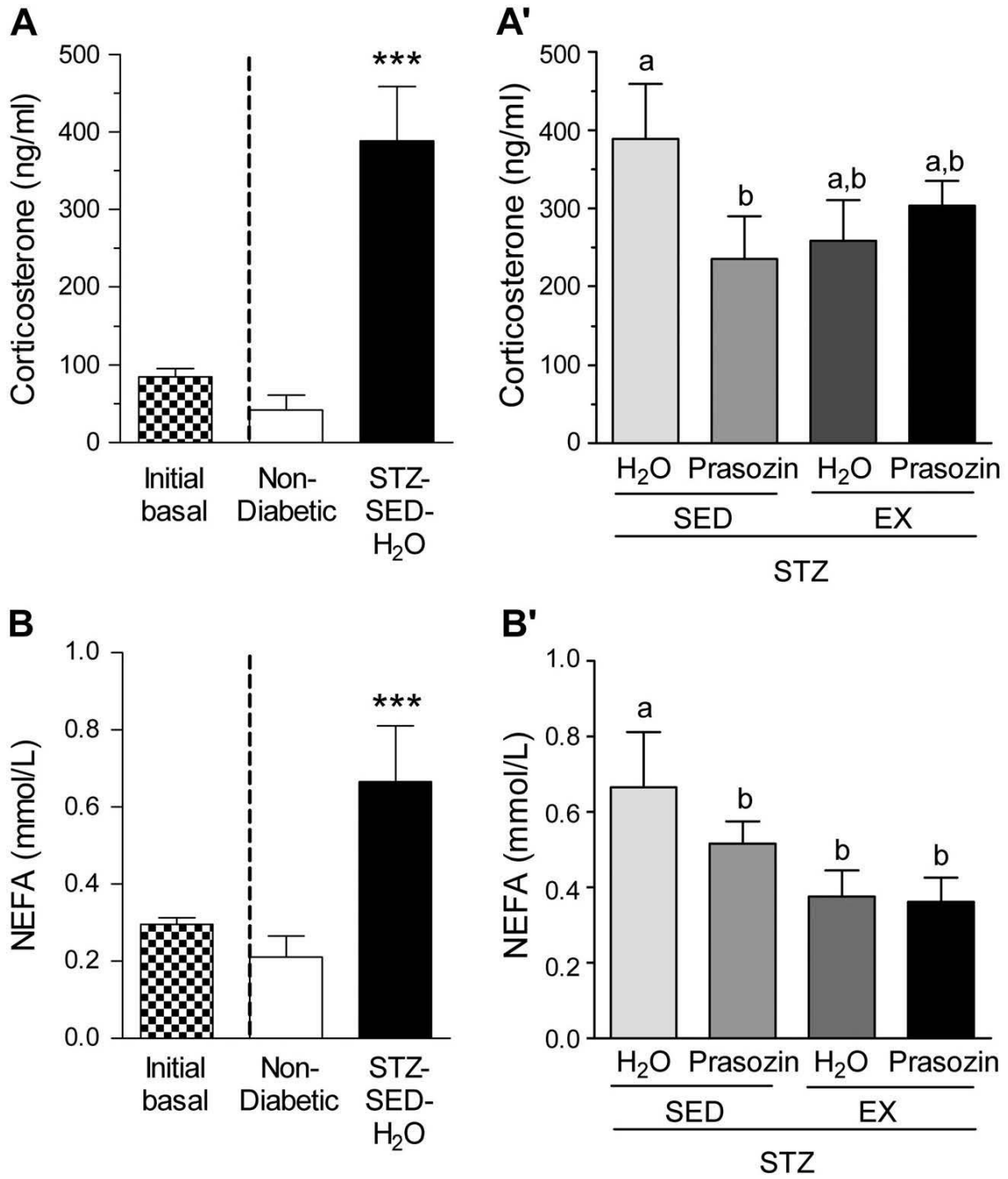
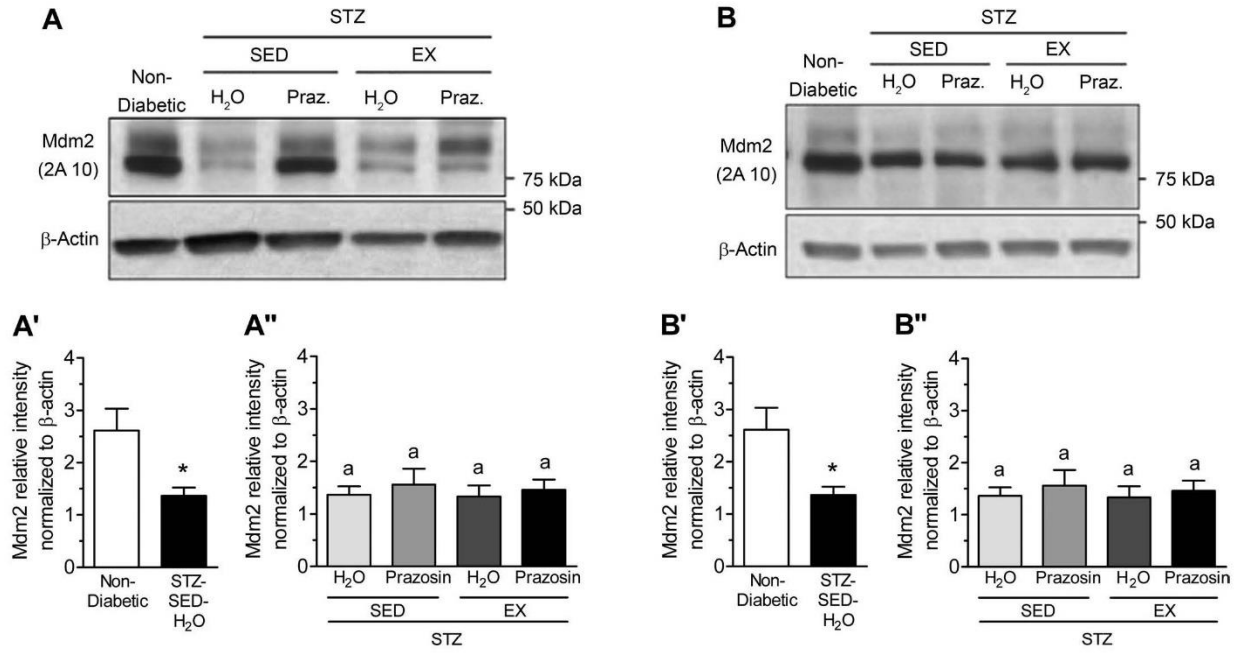


Figure 14.6

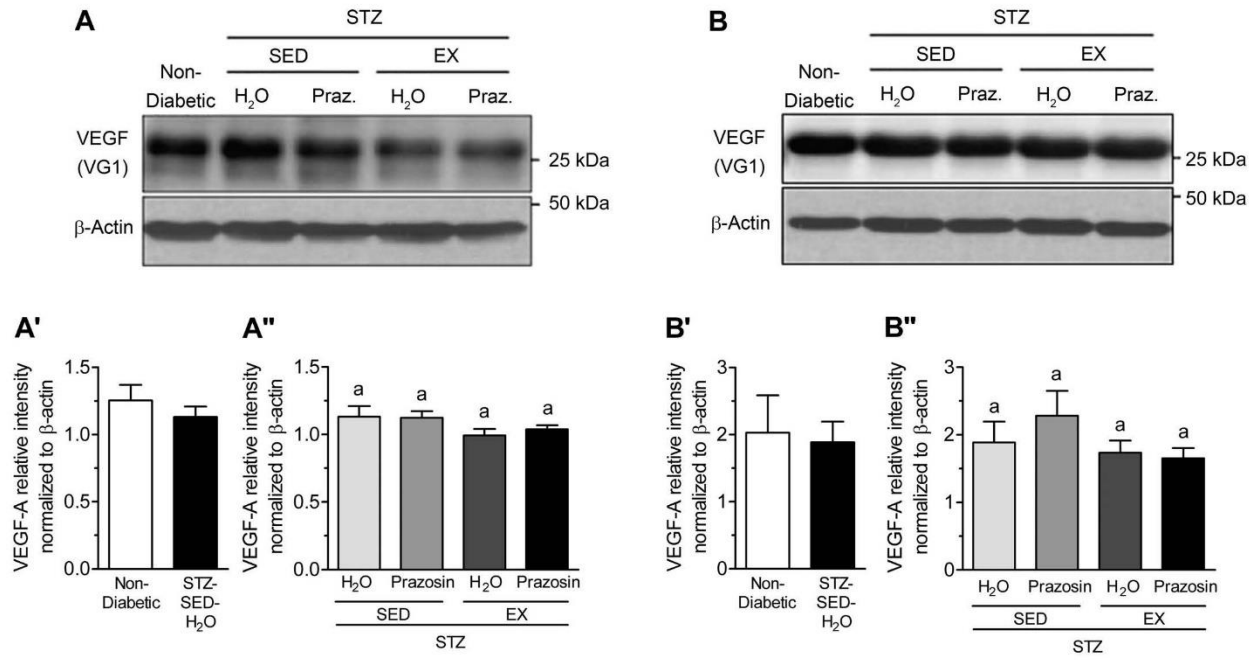


**Figure 14.7**





**Figure 14.8**



**Figure 14.9**

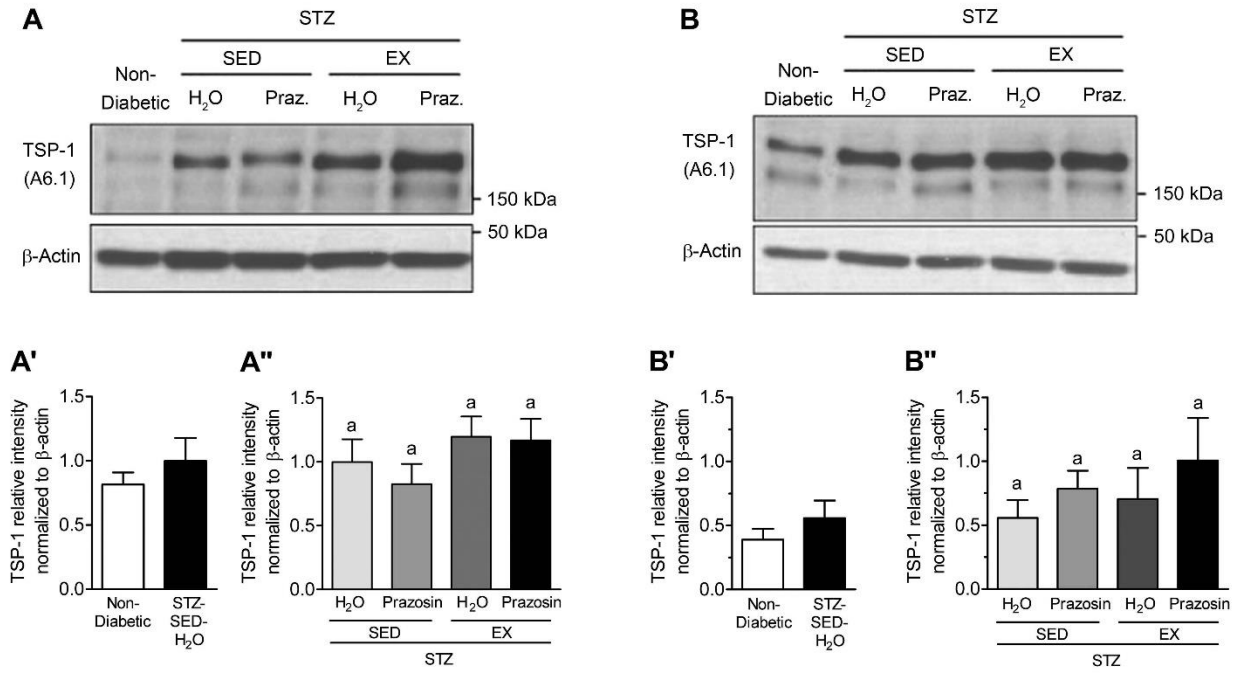


Figure 14.10

