Angioadaptive Allies: Examining the Relationship between Endothelial Cells and Human Skeletal Muscle Myofibroblasts

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ABSTRACT

Angioadaptation is the ability of capillaries to adapt to physiological changes. This is influenced by interactions between endothelial cells and supporting cells such as myofibroblasts. We examined a possible pro-angiogenic paracrine interaction between primary human dermal microvascular endothelial cells (HDMECs) and newly identified myofibroblast (MyoFib) progenitors in human skeletal muscle, and how this interaction may be altered under hyperglycemic conditions. Human skeletal muscle Myofib progenitor cells (CD90+) were sorted by FACS and differentiated into MyoFib using TGFβ under normo- (NG) or hyperglycemic (HG) conditions. The expression level of 55 angioadaptive proteins was measured by proteome array in the conditioned media. HDMECs were then treated with the conditioned media. VEGF-A, TSP-1 mRNA expression, and VEGF-A, TSP-1, p38, p-p38, and p-VEGF-R2 protein levels were measured by qPCR and western blot respectively. HDMEC migration was evaluated in the Boyden Chamber assay. HDMECs were also subjected to chronic HG and analyzed. The secretome from differentiated MyoFibs was enriched in pro-angiogenic factors compared to CD90+ cells. VEGF-A and p-p38 expression was increased in HDMECs when treated with MF secretome and their migration was stimulated. In contrast, CD90+ cell differentiation under HG resulted in decreased pro-angiogenic factor secretion in MF secretome and reduced EC migration. HDEMCs under HG had reduced migration and p-VEGF-R2. MyoFib exert some pro-angiogenic stimulation on HDMECs compared to progenitor cells. This pro-angiogenic effect is attenuated when cell differentiation occurs under HG.

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ABBREVIATIONS

αSMA – Alpha Smooth Muscle Actin

Ang-1 – Angiopoietin 1

CD – Cluster of Differentiation

ECGS – Endothelial Cell Growth Supplement

ECM – Endothelial Cell Media

ET-1 – Endothelin 1

FACS – Fluorescence Activated Cell Sorting

FBS – Fetal Bovine Serum

HB-EGF – Heparin Binding EGF-Like Growth Factor

HLA – Human Leukocyte Antigen

HAMEC – Human Adipose Microvascular Endothelial Cell

HDMEC – Human Dermal Microvascular Endothelial Cells

HG – Hyperglycemic/ High Glucose

HPRT - Hypoxanthine-Guanine Phosphoribosyltransferase

HRMEC – Human Retinal Microvascular Endothelial Cell

HUVEC – Human Umbilical Microvascular Endothelial Cell

ISCT – International Society for Cellular Therapy

MAPK – Mitogen Activated Protein Kinase

MMP – Matrix Metalloproteinase

MSC – Mesenchymal Stem Cell

MyoFib – Myofibroblast

NG – Normoglycemic/ Normal Glucose

P-VEGF-R – Phosphorylated Vascular Endothelial Growth Factor Receptor

PDGF-AA – Platelet Derived Growth Factor (AA-chain)

PGF – Placental Growth Factor

ROS – Reactive Oxygen Species

SP1 – Sphingosine-1-phosphate

SP1R – Sphingosine-1-phosphate Receptor

TGFβ – Transforming Growth Factor Beta

TIMP – Tissue Inhibitor of Metalloproteinases

TSP-1 – Thrombospondin-1

VEGF – Vascular Endothelial Growth Factor

VPF – Vascular Permeability Factor

LITERATURE REVIEW

Introduction

The microcirculatory system is critical to the maintenance and health of all tissues in the human body. It relies on capillaries, the smallest vessels, responsible for the exchange of oxygen, nutrients, and metabolic waste between the blood and cells. The skeletal muscle tissue is not an exception, and its capillary network has been well established as a key determinant of its function (Hudlicka, Brown, & Egginton, 1992).

Capillaries are composed of a single layer of endothelial cells. They serve as ideal sites for the exchange of oxygen and waste due to the minimal diffusion distances they provide. August Krogh was the first to suggest that each capillary can supply oxygen to a given cylindrical area of tissue surrounding the capillary in question (Krogh, 1919). Although Krogh's idea has since been expanded and elaborated on, the notion that a capillary supplies a fixed area still serves as the foundation for models of capillary function today (Egginton & Gaffney, 2010). Consequently, capillary networks are consistently aiming to parallel changes that occur within the tissue they supply.

Over the last few decades, the role and complexity of the microcirculatory system has been uncovered and defined. This has changed the dogma that capillaries simply formed a permissive barrier to instead the notion that their organization is dynamic, complex, and very reactive to changes within the microenvironment. Such functional plasticity can lead to the maintenance of capillaries, their regression, or their growth (angiogenesis), which is now referred to as angio-adaptation (Olfert & Birot, 2011; Roudier et al., 2010). Angiogenesis is a complex process, requiring the coordination of

a large number of signals and cell types. At the onset of sprouting angiogenesis, endothelial cells are liberated from the surrounding basement membrane. The extracellular matrix (ECM) requires proteolytic breakdown mediated by matrix metalloproteinases (MMPs) (Potente, Gerhardt, & Carmeliet, 2011). This allows freedom for endothelial cells to proliferate and migrate under the influence of angio-adaptive signals. Three major mechanisms are involved in endothelial migration during angiogenesis, including; chemotaxis, the directional migration toward a concentration gradient of soluble factors, haptotaxis, the directional migration toward a gradient of fixed ligands, and mechanotaxis, the directional migration generated by mechanical forces (Lamalice, Le Boeuf, & Huot, 2007). Vessel fusion, maturation, and stabilization takes place as new ECM components are deposited, and mural cells such as pericytes are recruited to the vessels (Carmeliet, 2003; Potente et al., 2011).

Angiogenesis plays a central role during embryogenesis, organ development, and responses to physiological challenges such as exercise. Insufficient vessel maintenance or growth is implicated in numerous conditions such as stroke, myocardial infarction, heart failure, and diabetes. Alternatively, excessive vessel growth can lead to cancer, ocular disorders, and various inflammatory disorders (Carmeliet & Jain, 2011; Hudlicka et al., 1992; Potente et al., 2011). Therefore, an appropriate and well coordinated angio-adaptive response is critical across the continuum of pathological to physiological challenges.

Angioadaptive Factors

A large myriad of angio-adaptive factors have been discovered to date. It is important to consider that the direction of angio-adaption (regression, growth, or stabilization) is determined by a delicate balance between pro- and anti-angiogenic factors. Changes on either side of the balance will ultimately shift the angio-adaptive characteristics of the micro-environment surrounding a given capillary network [Figure 1]. While there are numerous factors that fall on each side of the angio-adaptive balance, the pro-angiogenic Vascular Endothelial Growth Factor (VEGF) and the anti-angiogenic Thrombosondin-1 (TSP-1) are two of the most well characterized and extensively studied (Olfert & Birot, 2011).

Vascular Endothelial Growth Factor is a 35 – 45 kDa homodimeric protein that was initially discovered because of its ability to increase permeability of blood vessels, and was subsequently named Vascular Permeability Factor (VPF) (Senger, Perruzzi, Feder, & Dvorak, 1986). VEGF was shortly after isolated by Leung and colleagues, and has since been the dominant pro-angiogenic factor in angiogenesis research (Leung, Cachianes, Kuang, Goeddel, & Ferrara, 1989). There are currently five known members in the VEGF family, including placenta growth factor (PIGF), and VEGF-A through VEGF-D. VEGF-A is believed to be the most influential in angiogenesis (Egginton, 2009). It has been shown that alternative splicing from the eight exon VEGFA gene can lead to different isoforms of VEGF-A in humans, differentiated by their varying molecular weights. The first isoform described, VEGF-A_{165}, is the predominant isoform of VEGF-A, and has been extensively investigated (Ferrara, Gerber, & LeCouter, 2003). Between 1989 and 2003, other isoforms including VEGF-A_{121}, VEGF-$A_{145,}$ VEGF-$A_{148,}$ VEGF-$A_{183,}$

VEGF-A_{189}, and VEGF-A_{206} were identified and shown to be generated by alternative splicing of exons 6 and 7 (Harper & Bates, 2008) . More recently, another alternatively spliced form of VEGF-A was reported, and termed VEGF-A_{xxx}b. These isoforms are believed to have lower affinities for VEGF receptors, competing with VEGF-A to negatively regulate angiogenesis (Harper & Bates, 2008; Shibuya, 2013).

VEGF-A is produced by endothelial cells, and host tissue cells such as skeletal muscle. VEGF-A plays a number of critical roles in angiogenesis, including stimulating endothelial cell proliferation, migration, and survival (Egginton, 2009; Ferrara, 2004). While VEGF is acting on endothelial cells from a number of paracrine sources, it also appears that autocrine VEGF stimulation is required for vascular homeostasis (Lee et al., 2007).

VEGF acts through a family of tyrosine kinase receptors, composed of four main regions; the extracellular domain that binds the ligand, transmembrane domain, tyrosine kinase domain, and carboxyl terminal region (Ferrara et al., 2003; Shibuya, 2006). There are currently three known largely endothelial cell specific receptor tyrosine kinases. VEGF-Receptor 1 (VEGF-R1) or fms-related tyrosine kinase 1 (Flt-1), VEGF-Receptor 2 (VEGF-R2) also known as kinase insert domain receptor (KDR) in the human and fetal liver kinase 1 (Flk-1) in the mouse, and VEGF-Receptor 3 (VEGF-R3) also known as (Flt-4). Although they have different biological roles, each VEGFR has an essential activity in angiogenesis, as VEGFR-1, VEGFR-2, and VEGFR-3 knockout mice are all embryonically lethal due to vascular defects (Lamalice et al., 2007).

VEGF-A primarily exerts its effects through VEGF-R1 and VEGF-R2 (Koch & Claesson-Welsh, 2012; Shibuya, 2006). Although VEGF-R1 has a much stronger

affinity for VEGF, VEGF-R2 is the primary pathway for VEGF-A signaling in endothelial cells, with its activation leading to survival, proliferation, and migration responses (Egginton & Gaffney, 2010; Koch & Claesson-Welsh, 2012).

Binding of VEGF-A to VEGF-R2 leads to receptor dimerization, and promotes auto/trans-phosphorylation of several sites on the VEGF-R2 intracellular domain (Koch & Claesson-Welsh, 2012). This further activates multiple intracellular transduction pathways through downstream mediators resulting in biological responses such as the mentioned proliferation, migration, survival, and permeability. For example, activation of tyrosine 1175 induces activation of the PI3K-AKT and ERK 1/2 pathways. Furthermore, tyrosine 1214 has been shown to activate the cell migration associated p38 MAPK pathway, which is tied to alterations in cell adhesion and actin dynamics, promoting cell migration (Koch & Claesson-Welsh, 2012; Lamalice et al., 2007; Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006; Rousseau, Houle, Landry, & Huot, 1997).

Anti-angiogenic or angiostatic factors have garnered much less attention than their pro-angiogenic counterparts. One of the most widely studied and well characterized anti-angiogenic factors is Thrombospondin-1 (TSP-1). TSP-1 is a large (~450 kDa) multifunctional homotrimeric glycoprotein whose actions are targeted at inhibiting angiogenesis (Iruela-Arispe, Luque, & Lee, 2004; Olfert & Birot, 2011). Thrombospondin-1 is produced by endothelial cells, as well as smooth muscle cells, fibroblasts, neutrophils, and macrophages (Bornstein, 1992; Iruela-Arispe et al., 2004; Olfert & Birot, 2011). TSP-1 is also found localized in the extracellular compartments of host tissues and organs, including skeletal muscle, cartilage, skin, and bone (Bornstein, 1992; Iruela-Arispe et al., 2004)

TSP-1 acts as an anti-angiogenic factor by inhibiting cell migration, and inducing endothelial cell apoptosis. These actions are mainly accomplished through 3 receptors; Cluster of Differentiation 36 (CD36), β1-integrin, and Cluster of Differentiation 37 (CD37) (Bonnefoy, Moura, & Hoylaerts, 2008; Iruela-Arispe et al., 2004; Olfert & Birot, 2011; Ren, Yee, Lawler, & Khosravi-Far, 2006). Receptors CD36 and β1-integrin both interact with VEGF-R2, forming complexes in the endothelial cell membrane, and appear to inhibit VEGF-A induced phosphorylation and activation of VEGF-R2 (Olfert & Birot, 2011; Zhang et al., 2009). TSP-1 has also been shown to inhibit angiogenesis without interacting directly with the endothelial cell. For example, TSP-1 can impair matrix metalloproteinase 9 (MMP-9) activation, therefore impairing VEGF-A release from the extracellular matrix, or directly bind to VEGF-A itself and promote its internalization and degradation (Olfert & Birot, 2011).

Skeletal Muscle & Angio-adaptaion

Skeletal muscle tissue makes up 40% of our body weight, and plays a key role in metabolic regulation and locomotion. Active muscles require increased supply of oxygen and nutrients, and removal of metabolic by-products (Hudlicka et al., 1992). Skeletal muscle angio-adaptation is tightly regulated by a balance between pro- and anti-angiogenic signals (Ferrara et al., 2003; Olfert & Birot, 2011). The previously detailed VEGF-A and TSP-1 are to date the most well characterized regulators of skeletal muscle angio-adaptation (Olfert & Birot, 2011). The use of transgenic animal models has shown that modulation of VEGF-A and TSP-1 affected muscle capillarization with direct functional consequences in terms of exercise capacity and performance. In 2006, Wager's group showed muscle-targeted deletion of VEGF in

mice resulted in a loss of two-thirds of the muscle capillaries, and had a severe impact on exercise capacity (Wagner, Olfert, Tang, & Breen, 2006). This work was followed up by studies that demonstrated muscle specific VEGF deficiency reduces exercise endurance, and that myocyte VEGF is required for exercise induced skeletal muscle angiogenesis (Olfert et al., 2009; Olfert, Howlett, Wagner, Breen, & Virginia, 2010). From an anti-angiogenic signaling perspective, Malek and Olfert have shown that global deletion of TSP-1 increases both skeletal muscle capillarity and exercise capacity (Malek & Olfert, 2009).

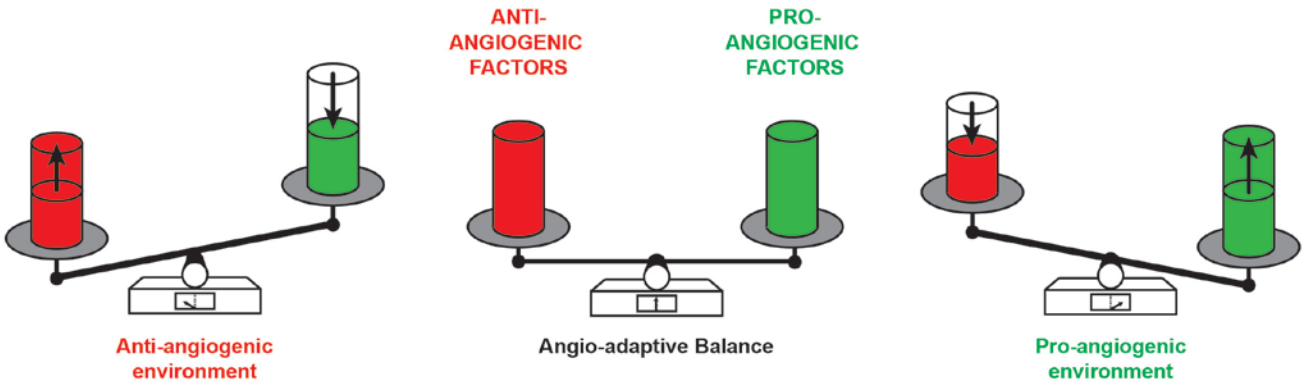

Figure 1. Angioadaptation as a balance between pro- and anti-angiogenic factors. The direction of angioadaptation is determined by a balance between pro- and anti- angiogenic factors. A predominance of pro-angiogenic signals such as VEGF will lead to a pro-angiogenic microenvironment, promoting capillary growth. A predominance of anti-angiogenic factors such as TSP-1 will instead promote capillary regression. When angio-adaptive factors are evenly balanced, the capillary network is maintained. It is important to note that changes can occur on either side of the balance independently, or accompany each other.

Physical exercise is a physiological stress that considerably challenges the balance between the blood supply and the myofibers' metabolic needs, and that therefore strongly stimulates skeletal muscle angiogenesis (Egginton, 2009). While one

single acute bout of exercise will alter the angio-adaptive balance at a molecular level, chronic exercise (i.e. training) will lead to the formation of new capillaries (Egginton, 2009). Conversely, under chronic pathological conditions such as obesity, diabetes, peripheral vascular disease (PVD), and chronic obstructive pulmonary disease (COPD), the angiogenic ability of the muscle is impaired and some capillary rarefaction can occur (Gouzi et al., 2013; Haas, Lloyd, Yang, & Terjung, 2012; Kivelä et al., 2008; Olfert et al., 2009). Muscle capillary rarefaction is not limited to pathological conditions, and can also occur in a physiological context such as muscle inactivity or detraining. Training-induced angiogenic remodeling was reversible in rats with 1 week of detraining, following a 10 week endurance training program (Malek, Olfert, & Esposito, 2010). Hypokinesia provides another example of non-pathological capillary rarefaction, as rats subjected to 9 day hindlimb unloading experience capillary rarefaction in the soleus muscle (Roudier et al., 2010).

What is striking about the research on skeletal muscle angio-adaptation is that the focus has been so far mostly restricted to the tissue level (i.e. the whole muscle) or to skeletal muscle endothelial cells alone. While the myofibers may be significant contributors of angiogenic stimuli during exercise conditions, the same may not be true under other physiological or pathological settings. Very few studies have addressed the role of other cell type contributors. Surrounding and supporting cell types also produce molecules that influence the microenvironment of a given capillary network (Davis & Senger, 2005; Gaengel, Genové, Armulik, & Betsholtz, 2009; Olfert & Birot, 2011; Potente et al., 2011). As noted earlier, VEGF-A and TSP-1 are secreted by a number of cell types in addition to the endothelial cell and myofibers, including fibroblasts, immune

cells, mural cells, and other supporting cell types (Bornstein, 1992; Ferrara et al., 2003; Olfert & Birot, 2011). While these paracrine interactions are essential, physical interactions between endothelial cells and various surrounding cell types are also paramount to the survival, growth, and maturation of capillaries. For instance, the stromal cells, which secrete and organize the extracellular matrix (ECM), provide structural support and organizational stability to the capillaries. Endothelial cells require adhesion to the ECM for migration, proliferation, and survival (Davis & Senger, 2005). Thus, the pro-angiogenic activity of endothelial cells is also highly dependent on paracrine, as well as physical interactions with their surrounding cells.

Hyperglycemia and the Microvasculature

Hyperglycemia is a hall mark of type I and type II diabetes (Bakker, Eringa, Sipkema, & van Hinsbergh, 2009). In physiological conditions, endothelial cells encounter blood glucose levels. in the range of ~3.6-5.8 mM, and cells exposed to glucose levels greater than 10 mM, in vitro or in vivo as in diabetes mellitus, are considered be in a high glucose condition (Popov, 2010). Diabetes is accompanied by a multitude of complications that negatively impact the integrity of the microvasculature and angiogenic activity (Beckman, Creager, & Libby, 2002; Martin, Komada, & Sane, 2003). High glucose levels disrupt endothelial cell homeostasis, leading to endothelial dysfunction and detrimental changes in the microvasculature, including (Popov, 2010). In diabetogenic conditions, angiogenic responses are enhanced in some tissues (retina and kidney), while they are impaired in other tissues, leading to defective wound healing, peripheral circulation, and coronary circulation (Moriya & Ferrara, 2014).

The Myofibroblast

Myofibroblasts were first identified as modified fibroblasts that expressed features of smooth muscle in the granulation tissue of healing wounds (Gabbiani, Ryan, & Manjo, 1971). Following injury, inflammatory cells and platelets secrete a host of pro-inflammatory cytokines such as transforming growth factor beta (TGF-β), which induce the differentiation of fibroblasts into myofibroblasts (Hinz, 2007; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Research has since been heavily focused on their role during wound healing. Myofibroblasts contribute to the wound healing process by producing a contractile force aimed at closing the wound margins. This ability to generate contractile forces relies on the expression of alpha smooth muscle actin (αSMA) (Hinz, 2007; Tomasek et al., 2002). Concurrently, myofibroblasts also contribute to the wound healing process by secreting several components of the ECM such as collagens of types I,III, IV, and V, thereby assisting in tissue remodeling and wound resolution (Hinz, 2007). Cells with myofibroblastic characteristics have been observed in a wide range of tissues and organs, including the skeletal muscle, liver, lung, and kidney among others (Dulauroy, Di Carlo, Langa, Eberl, & Peduto, 2012; Hao et al., 2006; Hinz et al., 2007; Li & Huard, 2002; Tomasek et al., 2002).

Although myofibroblasts play an undeniable role in successful healing, perpetuated activity can lead to negative consequences. Persisting infection, genetic disorders such as muscular dystrophy, or ageing, can lead to sustained chronic inflammation causing persistence of myofibroblast activity. This can lead to excessive deposition of ECM components and a stiffening and/or scarring of tissues, termed fibrosis (Hinz, 2007; Wynn, 2008).

Myofibroblast precursor cells are recruited from different sources depending on the tissues to be remodeled. The main progenitor population appears to be locally residing fibroblasts, however, pericytes, smooth muscle cells, bone marrow derived circulating cells, and epithelial cells have been shown to serve as important myofibroblast precursor populations (Hinz et al., 2007).

Myofibroblasts and Angiogenesis

Recent studies have pointed out an important role for the myofibroblast in angiogenesis (Daigle, Despatis, & Grenier, 2013). This is strengthened by the fact that pericytes, ubiquitous perivascular cells, have recently been shown to be an important cell progenitor population for myofibroblasts, and providing explanation of their presence in the vicinity of capillaries within virtually all vascularized tissue (Desmoulière, Guyot, & Gabbiani, 2004; Greenhalgh, Iredale, & Henderson, 2013; S.-P. Wong et al., 2015). For example, in addition to fibroblasts, activated pericytes have been shown to detach from local capillaries, migrate to sites of injury, and differentiate into myofibroblasts (S.-P. Wong et al., 2015). For this reason, studies have examined the possible paracrine relationship between endothelial cells and myofibroblasts. Endothelial cells have been to shown to proliferate faster when incubated with media conditioned by myofibroblasts (Nicosia & Tuszynski, 1994; Villaschi S, 1994). Cardiac and tumor associated myofibroblasts also produce and release various pro-angiogenic cytokines such as the VEGF-A (Chintalgattu, Nair, & Katwa, 2003; Vong & Kalluri, 2011). Interestingly, the production of VEGF-A by dermal myofibroblasts is significantly higher than from undifferentiated fibroblasts (Mayrand et al., 2012). This reinforces the

idea that myofibroblasts could play an important angio-adaptive role by modulating pro- and anti-angiogenic signals toward the endothelial cell.

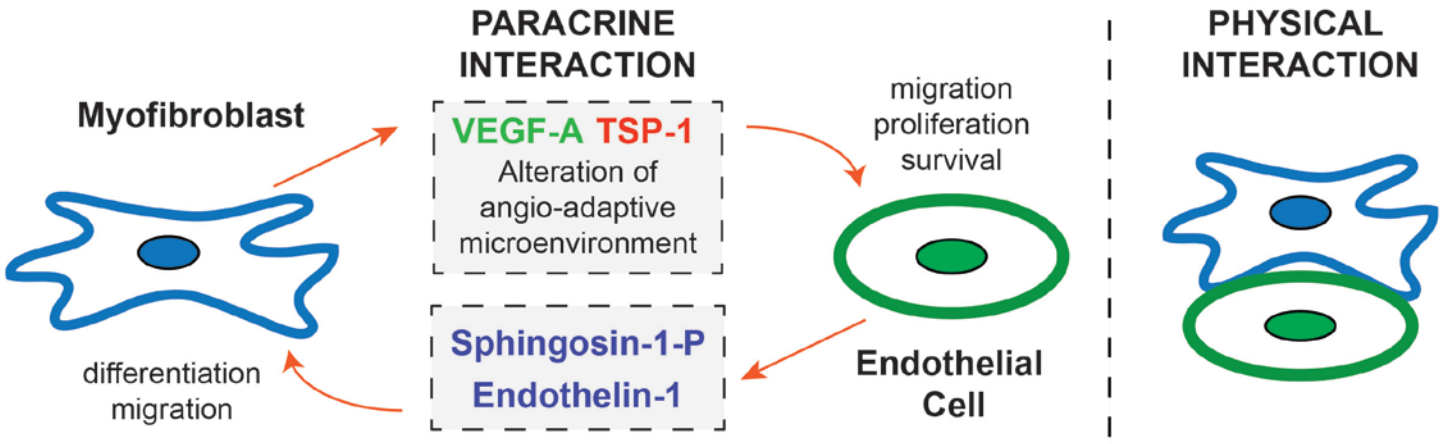

Figure 2. Reciprocal angio-adaptive relationship between the myofibroblast and endothelial cell. Myofibroblasts and endothelial cells appear to exist in a reciprocal paracrine and physical relationship that may have consequences on the functionality and viability of each cell type. Such interaction may be disrupted under pathological conditions.

This paracrine relationship can also be viewed from the perspective of the endothelial cell. For example, endothelial cells secrete endothelin-1 (ET-1), which promotes the differentiation of dermal fibroblasts into myofibroblasts during wound healing (Hinz, 2007; Leask, 2010; Villaschi S, 1994). Thus, these works strongly point out the existence of an active and reciprocal paracrine relationship between the myofibroblast and endothelial cell populations [Figure 2].

Several studies also suggest the existence of a physical relationship between these two cell types (Koike et al., 2004; Mayrand et al., 2012). Using co-culture experiments, it has been shown that mesenchymal cells and endothelial cells can lead to the formation of vascular tubes that are larger and at a higher density than tubes

obtained with endothelial cells only (Black, Berthod, L'heureux, Germain, & Auger, 1998; Koike et al., 2004; Villaschi S, 1994). Interestingly, the stability of these vascular tubes was also maintained for a longer period of time compared to tubes formed in cultures of endothelial cells alone (Mayrand et al., 2012; Nicosia & Tuszynski, 1994). It was also shown that when dermal myofibroblasts were co-cultured with endothelial cells, they maintained their differentiation into myofibroblasts for a longer period of time as shown through the expression of αSMA (Mayrand et al., 2012). In addition, the physical interaction between the two cell populations also promotes the production of type IV collagen from myofibroblasts, which is found in the supportive basement membrane of the vessels, providing vessel stability (Davis & Senger, 2005; Villaschi S, 1994). An *in vivo* study by Hansen et al. showing stretch-induced angiogenesis in skeletal muscle demonstrated a higher density of <10μm diameter vessels with αSMA-positive cells with respect to controls. Microscopic analyses provided evidence that growth of vessels in stretched muscles appeared to involve the migration of cells in the vicinity of capillaries, some of which were embedded in the capillary basement membrane. These mesenchymal cells, believed to be fibroblasts, migrated towards the capillaries before transforming into αSMA expressing pericytes or smooth muscle cells (Hansen-Smith, Egginton, Zhou, & Hudlicka, 2001). It is worth hypothesizing if these migrating cells share myofibroblastic characteristics.

Transformation of fibroblasts/pericytes into myofibroblasts involves the presence of other inflammatory factors such as Sphingosine 1-phosphate (S1P), a bioactive sphingolipid derived from sphingomyelin. S1P is implicated in the regulation of number of biological functions, including cell growth, migration, and inflammation. S1P acts

mainly through five G-protein coupled receptors, named S1PR1 - R5. In fibroblasts, SIP and S1PR1 are involved in cell migration and differentiation into myofibroblasts (Hamidi, Schäfer-Korting, & Weindl, 2014). Interestingly, it was demonstrated that S1P could accelerate the neo-vascularization process in diabetic mice and improve wound healing (Kawanabe, Kawakami, Yatomi, Shimada, & Soma, 2007). Although known to favour VEGF-A secretion by endothelial cells (Heo, Park, Kim, Kim, & Oh, 2009; Sun, Wei, Xu, Xu, & Zhang, 2010), it is appealing to propose that it could exert a similar effect in myofibroblasts that also secrete VEGF-A. Thus, this physical relationship between endothelial cells and myofibroblasts seems to play an equally important role as the paracrine cross-talk in an angiogenic context. Hence, identifying and targeting proper progenitors or differentiated myofibroblasts could provide benefits to angiogenesis.

Mesenchymal Stem Cells

Adult human stem cells have been derived from numerous tissues. They usually lack tissue specific characteristics, but can differentiate into specialized cells with phenotypic differences from their original precursor. It is generally thought that these stem cells residing in adult tissues serve as reservoirs, which can be mobilized and differentiated in response to the signals produced by wound and disease conditions (Barry & Murphy, 2004; Krampera, Pizzolo, Aprili, & Franchini, 2006).

Mesenchymal stem cells (MSCs) were first identified by Friedenstein and Petrakova in 1966, who isolated bone-forming progenitor cells from rat marrow (Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966). Numerous groups have confirmed and expanded on Friendenstein's initial findings, showing that human bone derived

MSCs have the ability to differentiate into numerous cell types such as osteoblasts, chondrocytes, adipocytes, and myoblasts (Barry & Murphy, 2004; Murray et al., 2014). MSCs were traditionally harvested from bone marrow, but have now been isolated from a number of human tissues including fat, skin, dental pulp, and muscle (Murray et al., 2014). Until now, most of the work that has been done aimed to characterize and expand these cells in vitro. Therefore, much of the current knowledge of MSCs is based on characterization and observations of behavior in culture (Murray et al., 2014).

Variations in methods of isolation, culture, and assays used to examine MSCs have made standardization of the nomenclature used in the field difficult. In 2006, the International Society for Cellular Therapy (ISCT) proposed the minimum criteria required to define MSCs (Dominici et al., 2006). They stated that cells must be plastic adherent, and must express cell surface antigens CD105, CD73, and CD90. Furthermore, these cells must not express the cell surface antigens CD45, CD34, CD14, CD11b, CD79α, CD19, or HLA-DR. Lastly, isolated cells are required to undergo tri-lineage differentiation into osteoblasts, adipocytes, and chondroblasts under defined culture conditions (Dominici et al., 2006; Murray et al., 2014). As discussed, MSCs have been shown to differentiate into other cell types while expressing other markers then those required in the ISCT guidelines. However, these 'extra' conditions are not required to define true MSCs (S.-P. Wong et al., 2015). Importantly, MCSs isolated from different organs and tissues exhibit unique features. The uniformity of MSCs from different origins has not been consistently demonstrated, even if they fulfill the ISCT criteria. MSCs from different origins have displayed differences in immunophenotype and secreted cytokine profile. Most of our understanding of MSCs is based on ex vivo

culture, and their in-vivo counterparts have remained elusive. Therefore, their native origin and physiological roles in vivo have been overlooked (Murray et al., 2014).

CD90+ Skeletal Muscle Derived Myofibroblast Progenitor

Dr. Grenier's group, at the University of Sherbrooke, have recently identified and characterized a novel human skeletal muscle resident myofibroblastic stem cell (Downey et al., 2015). These CD105, CD73, CD90 positive cells (referred to as CD90+ progenitors) are capable of differentiating into myofibroblasts upon stimulation with transforming growth factor beta (TGFβ). Myofibroblastic differentiation of these progenitors was confirmed by measuring the expression of αSMA and collagen type I at the gene and protein levels, as well as cell functionality through contractility. Seeing as how most research examining the relationship between myofibroblasts and endothelial cells has been limited to myofibroblasts of dermal and tumor origin, we saw the identification of these skeletal muscle progenitors as an exciting opportunity to explore their possible relationship with endothelial cells.

Conclusion

As discussed, much of the research in skeletal angiogenesis has been limited to looking at the whole muscle, or the endothelial cells themselves. Much less work has been done examining other surrounding and supporting cell types that could play an important role in influencing the microenvironment around a capillary, and the roles of stromal cells in angiogenesis has received too little attention. Concurrently,

myofibroblasts have been shown to play an important role in angiogenesis, however, nearly all of the work done exploring this relationship has been in a wound healing context with dermal myofibroblasts, or cancer setting with tumor myofibroblasts. Lastly, the physiological roles of potential MSC progenitors and their differentiated forms have often been overlooked. The aim of my project was to simultaneously tackle each of the three issues above by examining the possible angio-adaptive relationship between endothelial cells, and a novel myofibroblast progenitor derived from human skeletal muscle.

OBJECTIVES

1. To establish if myofibroblasts differentiated from newly discovered human skeletal muscle derived CD90+ progenitors have angio-adaptive properties through a paracrine relationship with endothelial cells.

2. To determine if the above angio-adaptive relationship is modified with metabolic disturbances such as hyperglycemia – a well established component of a diabetogenic model for endothelial cells.

HYPOTHESIS

1. Differentiated myofibroblasts will display a stronger pro-angiogenic activity compared to their CD90+ progenitor cells.

2. Hyperglycemic differentiation of CD90+ progenitors will negatively modify the pro-angiogenic properties of differentiated myofibroblasts.

MATERIALS AND METHODS

Human tissue collection and myofibroblast isolation, culture, differentiation, and conditioned media collection were performed by Dr. Grenier's group at the University of Sherbrooke.

Human Tissue

Healthy human skeletal muscle tissue samples (gracilis and semitendinosus) were obtained from patients (34 ± 8 years of age; 54% male and 46% female) undergoing anterior cruciate ligament reconstruction surgery. The samples were collected following resection surgery. The protocols were approved by the Centre Hospitalier de l'Université de Sherbrooke Ethics Committee (#11-122 and #13-164), and written consent was obtained from all patients.

CD90+ Progenitor Cell Isolation and Culture

Carefully dissected skeletal muscle samples were minced and then digested for 30 min at 37°C with 1 mg/mL of collagenase type I (Sigma) in DMEM containing 10% FBS. The tissue slurry was diluted with medium, passed through 70-µm and 40-µm cell strainers (Becton Dickenson), and centrifuged at 325g for 6 min at 4°C. Primary human cells were seeded in tissue culture plates coated with Mesencult-SF® attachment substrate and were expanded as adherent cells in Mesencult-XF® medium (StemCell Technologies). After 7 days, an average of $7x10^5$ adherent cells were recovered per gram of tissue. The cells were trypsinized at 80% confluence and were centrifuged and

resuspended in Mesencult-XF® medium as first passage cells, with fresh medium changes every 3-4 days. The cells were sub-cultured at a density of $4x10^3$ cells/cm^2.

Fluorescence-Activated Cell Sorting

First passage cells were detached with the Accutase™ Cell Detachment solution (BD Biosciences), centrifuged, and resuspended at ~$1x10^6$ cells per ml in cold sorting buffer (PBS, 1 mM EDTA, 25 mM HEPES, pH 7.0, 1% FBS). The cells were incubated for 20 min on ice, according to the manufacturers' instructions. During the cell sorting experiment, live cells were distinguished from dead cells using LIVE/DEAD® Violet Viability/Vitality kits (Invitrogen). Fluorescence was compensated using BD CompBeads Set Anti-Mouse Ig, κ (BD Biosciences). The cells were sorted using a BD FACSAria™ cell sorter (BD Biosciences) equipped with four lasers and a 100-µm nozzle set at 20 psi. Sorting gates were defined based on unstained controls. The cells were analyzed using FlowJo 7.9 software (Treestar Inc.). A population of unsorted cells was used as a control.

Myofibroblast Differentiation

The cells were seeded at a density of 8×10^3 cells per well in 24-well collagen-coated (Millipore) plates (4000 cells/cm2) in Mesencult-XF® medium and incubated at 37 °C in a CO2 incubator until they reached confluence. To stimulate myofibroblastic differentiation, CD90+ progenitor cells were incubated in myofibroblast differentiation medium (DMEM 2% HS, 1% antibiotics) containing 25 ng/ml TGFβ for 5 days. The TGFβ was omitted for the un-stimulated controls. Differentiation media was either

normoglycemic (5 mM glucose) or hyperglycemic (25 mM glucose) and were changed twice every 3 or 4 days.

Endothelial Cell Culture

Primary human dermal microvascular endothelial cells were purchased from ScienCell Research Laboratories (Cat. 2000, Lot 8219, Carlsbad, CA, U.S.A.). Cells were cultured at 37^{o}C and 5% CO_2, on T75cm^2 coated with 50µg/mL collagen I, from rat tail (Gibco, cat. A10438-01, Burlington, ON, Canada) in 0.02 M acetic acid (Sigma-Aldrich, cat. 320099, Oakville, ON, Canada). Cells were maintained in Endothelial Cell Media (ECM, ScienCell, Cat. 1001, Carlsbad, CA, U.S.A.) supplemented with 5% fetal bovine serum (ScienCell, Cat No. 0025, Carlsbad, CA, U.S.A.), 1% endothelial cell growth supplement (ECGS, ScienCell, Cat. 1052, Carlsbad, CA, U.S.A.), and antibiotic solution containing 100 U/mL of penicillin and 100 µg/mL streptomycin (ScienCell, Cat. 0503, Carlsbad, CA, U.S.A.).

Conditioned Media (secretome) Collection

CD90+ & Myofibroblast conditioned media:

Cells were seeded and allowed to grow to 80% confluence. CD90+ cells were stimulated with normo- or hyperglycemic differentiation medium for 72 hours. Cells were rinsed with PBS (Gibco, Cat. A10438-01, Burlington, ON, Canada), and serum free medium with 1% BSA was added. Cells were allowed to incubate for 72 hours before the supernatant was collected. The supernatant was centrifuged at 5000g for 5 minutes to remove cellular debris, and the conditioned media was collected.

HDMEC conditioned media:

HDMEC were cultured under normo- (5 mM glucose) or hyperglycemic (25 mM glucose) conditions for 12 days (3 passages). Cells were then seeded and allowed to grow to 70% confluence. Cells were rinsed with PBS (Gibco, cat. A10438-01, Burlington, ON, Canada), and serum free endothelial cell medium (ECM) (ScienCell, Cat. 1001, Carlsbad, CA, U.S.A.) supplemented with 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich, cat. A7906, St. Louis, MO, U.S.A) was added. The cells were allowed to incubate for 24 hours before the supernatant was collected. The supernatant was centrifuged at 5000g for 5 minutes to remove cellular debris, and the conditioned media was collected.

Protein Concentration Determination

Determination of total protein concentration was done using the Bicinchoninic Acid Assay (BCA). The BCA solution was prepared from Bicinchoninic Acid (Sigma-Aldrich, cat. B9643, Oakville, ON, Canada) and Copper II Sulphate (Sigma-Aldrich, cat. C2284, Oakville, ON, Canada) solutions in a 20:1 ratio, respectively. Samples to be analyzed were loaded in triplicate into a 96-well round bottom plate (Sarstedt, cat. 82.1581, Newton, NC, U.S.A.). Samples were compared to a standard curve, plotted by using dilutions of a stock 2 mg/ml solution of Bovine Serum Albumin (Sigma-Aldrich, cat. A7906, St. Louis, MO, U.S.A). Plates were incubated at 37°C for 30 minutes and the absorbance was read at 562 nm on a plate reader (Wallack Victor3 1420 Multilabel Counter, PerkinElmer).

Angiogenesis Proteome Profiler Array

The assay was run according to the manufacturer's instructions (R&D Systems, cat. ARY007, Burlington, ON, Canada). Conditioned media samples were incubated with nitrocellulose membranes pre-probed with primary antibodies against 55 positive and negative regulators of angiogenesis, with each antibody being spotted in duplicate on the membranes. Each membrane was incubated with 300 µg of total proteins from conditioned media samples. Conjugated protein spots were visualized by incubating each membrane with a cocktail of biotinylated detection antibodies, streptavidin-horseradish peroxidase, and visualized using Millipore Luminata™ HRP Chemiluminesence Detection Reagents (cat. WBLUC0100, WBLUR0100, WBLURUF0100, Thermo Scientific, Nepean, ON, Canada). Protein spots were quantified using a Kodak Imaging station 4000MM Pro with Carestream software.

Endothelial Cell Migration

One day prior to the cell migration assay, cells were trypsinized, collected, counted, and re-plated. Migratory activity was measured using a Boyden chamber assay (Neuro Probe Inc, cat. AP48, Gaithersburg, MD, U.S.A.). A Polycarbonate membrane filter with 8µm pores (Neuro Probe, Inc., cat. PFB8, Gaithersburg, MD, U.S.A.) was coated with 50µg/mL collagen (Gibco – Life Technologies, cat. A10438-01, Burlington, ON, Canada) in 0.02 M acetic acid (Sigma-Aldrich, cat. 320099, Oakville, ON, Canada) and allowed to incubate overnight prior to the assay at 4^{o}C.

Cells were used between passages 5 to 10. Cells were collected and resuspened in ECM (ScienCell, cat. 1001, Carlsbad, CA, U.S.A.) supplemented with 1% Fetal

Bovine Serum (ScienCell, cat. 0025, Carlsbad, CA, U.S.A.) at a concentration of 440 cells/µl. Wells of the bottom chamber were loaded with 28 µl of either non-stimulating, stimulating, or conditioned media. Stimulating medium consisted of ECM (ScienCell, cat. 1001, Carlsbad, CA, U.S.A.) supplemented with 10% Fetal Bovine Serum (ScienCell, Cat. 0025) and 5% Endothelial Cell Growth Supplement (ScienCell, cat. 1052, Carlsbad, CA, U.S.A.), while non-stimulating medium consisted of ECM (ScienCell, cat. 1001, Carlsbad, CA, U.S.A.) supplemented with 1% Fetal Bovine Serum (ScienCell, cat. 0025, Carlsbad, CA, U.S.A.). These two conditions were used respectively as positive and negative controls. Wells of the top chamber were filled with 50 µl of cell suspension (440 cells/µl). The assembled and filled chamber was placed in the incubator at 37^{o}C and 5% $CO_{2,}$ and cells were allowed to migrate for 4.5 hours. Following migration, the membrane was incubated in 20 mL cold (4 ^{o}C) methanol (Caledon Laboratory Chemicals, cat. 6700-1, Georgetown, ON, Canada) for 10 minutes at room temperature. The membrane was then washed 3 times with cold (4 ^{o}C) double distilled water and stained using 2 mL of Giemsa stain (Sigma-Aldrich, cat. GS500, Oakville, ON, Canada) diluted in 20 mL double distilled water, and allowed to incubate for 30 minutes at room temperature. The membrane was briefly destained in double distilled water and mounted on a microscope slide. A damp cotton swab was used to carefully scrape the non-migrated cells from the top of the membrane. Cells were counted under 40X magnification, with 4 counts performed per well. Counting was expressed as cells per field of view.

Gene Expression

Primary human microvascular endothelial cells were treated with CD90+ progenitor or myofibroblast conditioned media for 16 hours, or grown under normo- and hyperglycemic conditions over a 12 days (3 passages). Following stimulation, RNA was collected using the Cell-to-cDNA II kit (Ambion - Life Technologies, cat. 1722, Burlington, ON, Canada). cDNA samples were analysed by Taqman real-time quantitative polymerase chain reaction (qPCR) with an ABI 7500 Fast PCR system (Invitrogen – Life Technologies, Burlington, ON, Canada) using qPCR master mix (Invitrogen – Life Technologies, cat. 11743, Burlington, ON, Canada) and Taqman probes targeted to VEGF-A, TSP-1, and Hypoxanthine-guanine phosphoribosyltransferase (HPRT), followed by quantification using comparative ΔΔCT analysis to determine relative mRNA expression. HPRT was used as a housekeeping gene.

Western Blots

Immunoblotting was carried out on protein extracts from human dermal microvascular endothelial cells that underwent treatment with conditioned media or normo/hyperglycemic culture conditions. Protein was extracted using lysis buffer containing mg/mL phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, 1 mmol/L NaF (Sigma-Aldrich, Oakville, ON, Canada), and 1X protease inhibitors (Complete Mini and PhosStop tablets from Roche Diagnostics, Laval, QC, Canada). Twenty micrograms of protein per sample, as determined by BCA, was prepared in a loading buffer containing 2.84 M β-mercaptoethanol, 0.28 M Sodium dodecyl sulfate, 2.69 M sucrose, 14 μM

bromophenol blue, and 0.1 M of 0.5 M Tris pH 6.8, before being boiled at 100°C for 6 minutes. Samples were loaded into 7.5-15% SDS polyacrylamide gel and separated by electrophoresis at 200V, at room temperature (please refer to Appendix A for details). Proteins were then transferred on to a 0.45 µm nitrocellulose membrane (Fisher Scientific, cat. 45004000, Toronto, ON, Canada) at 4°C for 70-80 minutes (please refer to Appendix A), at 100 V, using the wet transfer method.

After blocking with 5% fat-free milk in tris buffer saline (TBS) containing 0.1% tween at room temperature for 45 min, the blots were probed overnight at 4°C with light agitation, with primary antibodies against the following proteins: vascular endothelial growth factor – A (VEGF-A) (clone VG-1, cat. 05-1117, Millipore, Temecula, CA, U.S.A.), thrombospondin-1 (TSP-1) (clone A6.1, cat. MS-421-P0, NeoMarkers – Thermo Scientific, Nepean, ON, Canada), phospho-VEGF receptor 2 (Tyr1175) (clone DB511, cat. 3770, Cell Signaling, Beverly, MA, U.S.A.), p38 MAPK (cat. 9212, Cell Signaling, Beverly, MA, U.S.A.), phospho-p38 (clone D-8, cat. sc-7973, Santa Cruz Biotechnologies, CA, U.S.A.), β-actin (clone C4, cat. sc-47778, Santa Cruz Biotechnologies, CA, U.S.A.), α/β Tubulin (cat. 2148, Cell Signaling, Beverly, MA, U.S.A.). β-actin and α/β-tubulin were detected as loading controls (Please see Appendix A for all antibody concentrations). Membranes were then probed for 60 minutes and at RT under light agitation with the following appropriate secondary antibodies diluted in 5% BSA: HRP conjugate Rabbit anti-mouse (cat. p0260, Dako, Mississauga, ON, Canada), or HRP conjugated Mouse anti-rabbit (cat. 7074, Cell Signalling, Pickering, ON, Canada).

Proteins were visualized using Millipore Luminata™ HRP Chemiluminesence Detection Reagents (cat. WBLUC0100, WBLUR0100, WBLURUF0100, Thermo Scientific, Nepean, ON, Canada) and the signals were detected using CL-Exposure™ Film (Thermo Scientific, Nepean, ON, Canada). Western blot images were digitized, and analyzed with Carestream Molecular Imaging software (Carestream Health, Rochester, NY, U.S.A.). Protein expression levels were normalized to loading control expression levels, and the respective ratios were compared between different groups.

Statistical analysis

Analyses were performed using Prism5 software (GraphPad). All data are presented as means ± S.E.M. T-tests were used to analyze the results of mRNA and protein expression for differences between the respective stimulatory conditions. One-way ANOVA and two-way ANOVA were used to analyze the results of the Boyden chamber migration assays. Differences between the groups were determined using Newman Keuls' post-hoc test. The results were considered to be statistically significant when $P \leq 0.05$.

RESULTS

Differentiated myofibroblasts express key angioadaptive factors.

Before exploring their potential angiogenic relationship with endothelial cells, we examined if myofibroblasts (MyoFib) differentiated from skeletal muscle derived CD90+ progenitors (CD90+) represented a source of angioadaptive factors. We specifically measured VEGF-A and TSP-1 gene expression by Taqman qPCR. While the myofibroblasts expressed VEGF-A and TSP-1 mRNA, there were no significant differences in expression whether the differentiation took place under normoglycemic or hyperglycemic conditions [Figure 4, VEGF: 0.94 ± 0.04 CD90+ vs. 1.11 ± 0.05 MyoFib, TSP-1: 1.12 ± 0.06 CD90+ vs. 1.19 ± 0.05].

Myofibroblast conditioned media (secretome) is enriched with pro-angiogenic factors compared to CD90+ progenitor cells' secretome, and stimulates human dermal microvascular endothelial cell migration.

We next wanted to assess the angio-adaptive profile of the secreted proteins from CD90+ progenitors and differentiated myofibroblasts. Conditioned media (secretome) was collected from CD90+ progenitors and differentiated myofibroblasts, and analyzed via a proteome profiler array (See Appendix 4 for full results). Myofibroblast conditioned media was characterized by an increased expression of several pro-angiogenic factors, including VEGF and angiopoietin-1, when compared to the conditioned media from CD90+ progenitor cells. Anti-angiogenic factors such as

TSP-1 and TIMP-1 appear to be secreted less in differentiated myofibroblasts [Figure 5].

Given the change seen in the angiogenic profile of the secretome following differentiation, we looked to examine the possible functional impact of the respective secretomes on HDMEC migration through the Boyden Chamber assay. HDMECs stimulated with myofibroblast secretome migrated 60% more than cells treated with CD90+ progenitor cell secretome, complementing the changes seen in the proteome array of the secretomes [Figure 6, 92.3 ± 3.6 % CD90+ vs. 146.8 ± 3.9 % MyoFib, $P \leq 0.001$].

HDMEC treated with myofibroblast conditioned media expressed increased levels of phosho-p38 and VEGF-A, with no change in TSP-1 or phospho-VEGF-R2.

With the changes seen in HDMEC functionality following treatment with conditioned media, we explored the possible changes in HDMEC molecular signalling that could be involved. We investigated phospho-p38 expression, as p38 MAP kinase activation by VEGF has been shown to mediate endothelial cell migration (Koch & Claesson-Welsh, 2012; Rousseau et al., 1997). HDMECs treated with myofibroblast secretome expressed a 73% increased levels of phospho-p38 protein (relative to total p38) when compared to HDMECs treated with CD90+ progenitor secretome [Figure 8, 0.15 ± 0.01 CD90+ vs. 0.26 ± 0.04 Myofib, *, $P \leq 0.05$]. We next examined HDMEC VEGF-A and TSP-1 mRNA and protein expression to gain a perspective of the autocrine angiogenic state of the endothelial cells exposure to the conditioned media. HDMEC treated with myofibroblast secretome had a 75% increase in VEGF-A mRNA

expression [Figure 7, 1.00 ± 0.03 CD90+ vs. 1.75 ± 0.19 MyoFib, **, $P \leq 0.01$], and a 110% increase in VEGF-A protein expression [Figure 7, 0.62 ± 0.07 CD90+ vs. 1.30 ± 0.13 MyoFib, *, $P \leq 0.05$]. HDMEC thrombospondin-1 mRNA [Figure 7, 1.00 ± 0.09 CD90+ vs. 1.01 ± 0.04 MyoFib] and protein expression [Figure 7, 0.61 ± 0.03 CD90+ vs. 0.50 ± 0.03 MyoFib] were unaltered following treatment with CD90+ or Myofib conditioned media. The change in protein levels was further represented as a VEGF to TSP-1 ratio, which increased by 153% in the HDMECs treated with the myofibroblast conditioned media [Figure 7, 1.02 ± 0.17 CD90+ vs. 2.58 ± 0.38 MyoFib, *, $P \leq 0.05$]. There was no change in phospho-VEGF-R2 protein expression between the two treatment conditions [Figure 8, 0.52 ± 0.17 CD90+ vs. 0.77 ± 0.11 MyoFib].

Differentiation of myofibroblasts under hyperglycemic conditions attenuates the pro-angiogenic profile of the secretome, and reduces the pro-migratory impact on HDMECs.

Conditioned media collected from myofibroblasts differentiated under hyperglycemic conditions (25 mM glucose) appeared to have an overall decrease in pro-angiogenic factors, including angiopoietin-1 and VEGF, with relative little change or increase in anti-angiogenic factors [Figure 9].

Given the changes in the proteome array, we examined if hyperglycemic differentiation had an impact on the secretomes' ability to induce HDMEC migration. There was no difference in the ability of progenitor CD90+ secretome to stimulate migration following normo- or hyperglycemic exposure [Figure 10, 103.3 ± 2.7% CD90+ NG vs. 103.8 ± 1.5 % CD90+ HG]. HDMEC stimulated with conditioned media collected

following normoglycemic differentiation migrated 49% more than those stimulated with CD90+ progenitor conditioned media, confirming what shown earlier in the results above [Figure 10, 103.3 ± 2.7% CD90+ NG vs. 155.1 ± 2.8 % Myofib NG - effect of differentiation, †††, $P \leq 0.001$]. When the conditioned media was collected following hyperglycemic differentiation, treated HDMECs had only a 10% increase in migration compared to CD90+ progenitor conditioned media [Figure 10, 103.8 ± 1.5 % CD90+ HG vs. 113.0 ± 2.1 % Myofib HG – Effect of differentiation, †, $P \leq 0.05$]. Importantly, this represents a 27% reduction in migration when comparing the stimulation by conditioned media collected following normoglycemic or hyperglycemic differentiation [Figure 10, 155.1 ± 2.8 % Myofib NG vs. 113.0 ± 2.1 % Myofib HG – Effect of HG, $$$, $P \leq 0.001$].

HDMECs treated with myofibroblast secretome collected following hyperglycemic differentiation had an increase in VEGF-A mRNA expression, with no change in VEGF-A, TSP-1, phospho-38, or phosho-VEGF-R2 protein levels.

HDMEC treated with myofibroblast secretome collected following hyperglycemic differentiation expressed 61% higher levels of VEGF-A mRNA compared to those treated with conditioned media following normoglycemic differentiation [Figure 11, 1.76 ± 0.19 MF NG vs. 2.83 ± 0.16 MF HG, ***, $P \leq 0.001$]. However, there was no change in VEGF-A protein expression between the two groups. [Figure 11, 1.30 ± 0.13 MF NG vs. 1.10 ± 0.09 MF HG]. There were also no significant differences in TSP-1 mRNA expression [Figure 11, 1.01 ± 0.04 MF NG vs. 1.03 ± 0.078 MF HG] or protein expression [Figure 11, 0.50 ± 0.03 MF NG vs. 0.58 ± 0.04 MF HG]. While there appeared to be a trend for a decrease in phospho-p38 (relative to total p38), there was

no significant difference between the HDMECs treated with the different conditioned media. [Figure 12, 0.26 ± 0.04 MF NG vs. 0.17 ± 0.04 MF HG]. Lastly, there was no significant difference in p-VEGF-R2 expression between the two groups [Figure 12, 0.77 ± 0.11 MF NG vs. 0.91 ± 0.18 MF HG].

HDMEC cultured under hyperglycemia have reduced migratory activity.

HDMEC cultured under hyperglycemic (25 mM) conditions over a 12 day period (3 passages) had a 13 % reduction in basal migration in response to the no-stimulation (non-stim) condition [Figure 13, 100.0 ± 0.7 % Non-stim NG vs. 87.4 ± 0.7 % Non-stim HG, ***, $P \leq 0.001$]. The ability of HDMECs to migrate in response to the stimulatory condition was also compromised. Cells under normal glucose and exposed to the stimulatory condition (stim) underwent a 82% increase in migration compared to the no stimulation group [Figure 13, 100.0 ± 0.7 % Non-stim NG vs. 181.7 ± 0.7 % Stim NG, ***, $P \leq 0.001$], however, HDMECs under high glucose, and exposed to the stimulatory condition only had a 65% increase compared to the respective non-stim group. This represents a 10% decrease in HDMECs ability to respond to the stimulatory condition within each respective glucose condition [Figure 13, 87.5 ± 0.7 % Non-stim HG vs. 144.2 ± 1.2 % Stim HG, †††, $P \leq 0.001$].

HDMEC cultured under hyperglycemia have unaltered VEGF-A and TSP-1 protein expression, but a decrease in phospho-VEGF-receptor 2 protein levels.

Following the changes in HDMEC migration with hyperglycemic conditions, we looked to investigate the autocrine angiogenic state of the cells. We again looked at VEGF-A and TSP-1 expression. VEGF-A mRNA [Figure 14, 0.45 ± 0.05 NG vs. 0.48 ± 0.05 HG] and protein [Figure 14, 0.23 ± 0.05 NG vs. 0.21 ± 0.03 HG] expression were unaltered following culture under hyperglycemia. Thrombospondin-1 mRNA expression was 71% higher in HDMEC cultured under hyperglycemia compared to those under normoglycemia [Figure 14, 0.55 ± 0.06 NG vs. 0.94 ± 0.10 HG, *, $P \leq 0.05$], however, protein levels were unaltered between the two culture conditions [Figure 14, 2.92 ± 0.23 NG vs. 2.84 ± 0.26 HG]. Given the lack of changes in these two angioadaptive factors, we examined the phosphorylation of VEGF receptor-2. Phoshpo-VEGF-R2 protein expression was 48% lower in HDMEC cultured under hyperglycemia compared to normoglycemia [Figure 14, 0.33 ± 0.04 NG vs. 0.17 ± 0.02 HG, **, $P \leq 0.01$].

DISCUSSION

As detailed in the introduction, too little attention has been given to potential role supporting cells types could play within skeletal muscle angio-adaption. Concurrently, in the investigation of mesenchymal stem cells, the focus has been on the characterization of cell types, while the physiological roles of MSC progenitor cells have often been overlooked (Murray et al., 2014). We looked to address both of these issues with our investigation of the possible angio-adaptive relationship between newly identified human skeletal muscle myofibroblast progenitor cells and endothelial cells.

We have shown that myofibroblasts acquire a proangiogenic profile when differentiated form their skeletal muscle derived CD90+ progenitor cells. This is in accordance with other publications suggesting a proangiogenic role for myofibroblasts derived from other tissues (Daigle et al., 2013; Mayrand et al., 2012; Vong & Kalluri, 2011). It is worth being highlighted the good correspondence between results obtained at the molecular level for the angio-adaptive proteome profiling and those examining aspects of cell functionality such as migration. Although only qualitative, the proteome array provided us with an interesting overview of the general angio-adaptive profile of secreted proteins from the differentiated myofibroblasts compared to their progenitors. In particular, it suggested a general increase in several well-established pro-angiogenic factors, and either a decrease or the maintenance of anti-angiogenic molecules when comparing the secretome from the CD90+ progenitors to that of the differentiated myofibroblasts. These results from the molecular profiling were in line with the functional analysis of primary endothelial cell migration. Cell migration was indeed significantly increased in response to stimulation with the myofibroblast secretome compared to

stimulation with CD90+ progenitor secretome. Considering how endothelial cell migration is a fundamental aspect of the angiogenic process, these results strongly support the hypothesis of a proangiogenic relationship between CD90+-differentiated myofibroblasts and primary endothelial cells.

Moreover, HDMEC express more proangiogenic VEGF-A when treated with myofibroblast secretome compared to CD90+ secretome, while HDMEC TSP-1 expression remains unaffected. This was shown to be true at both the mRNA and protein level. These results provide an overall view of the angio-adaptive state of the endothelial cell itself. The shift in the direction of the pro-angiogenic side of the balance is further illustrated by the significant increase in the VEGF to TSP-1 ratio, a measure that has been previously shown to correlate well with muscle angio-adaptive patterns (Roudier et al., 2010). While it is unclear what the specific roles of endothelial cell produced endogenous angio-adaptive factors are during angio-adaptation, autocrine VEGF-A signalling has been shown to be required for vascular homeostasis (Lee et al., 2007).

We further investigated endothelial signaling pathways the myofibroblast conditioned media could be impacting to promote changes seen in cell migration. The expression of phospho-VEGF receptor-2 and phospho-p38 were prime candidates given the central role they play in endothelial cell migration signalling following VEGF stimulation (Koch & Claesson-Welsh, 2012; Lamalice et al., 2007). A significant increase was seen in phospho-p38 expression in the HDMECs treated with the myofibroblast secretome. This was consistent with the changes seen in endothelial cell migration, and confirmed that the signalling may be occurring through VEGF-Receptor

activation and subsequent p38 MAPK signalling. However, there was no change in p-VEGF-R2 expression in the same cells. Although there was trend for an increase, the 16 hour treatment with conditioned media may have been too long of a time point to capture changes in the phosphorylation of the receptor, while changes in p38 phosphorylation may have persisted.

We have also shown that the pro-angiogenic profile of the differentiated myofibroblasts was negatively altered when differentiation occurred under hyperglycemic conditions. We chose to examine the impact of hyperglycemic stress on the myofibroblasts as it considered the hallmark of type 2 diabetes (Bakker et al., 2009; Popov, 2010). While it is important to recognize that type 2 diabetes presents a number of other detrimental complications such as dyslipidemia and hyperinsulinemia, hyperglycemia served as a useful and widely used point of departure to examine the myofibroblast–endothelial cell relationship under pathological conditions. It has been shown that the differentiation of myofibroblasts appears to be altered under diabetogenic conditions (Fowlkes et al., 2013). However, this study utilized cardiac myofibroblasts, and focused on the pro-fibrotic consequences rather than angio-adaptive aspects.

The qualitative analysis of the proteome profiler suggested that the myofibroblasts differentiated under hyperglycemia presented a decreased release of pro-angiogenic factors in their secretome compared to cells differentiated under normoglycemic conditions. These results were quantitatively supported by the results from the cell migration assay, as HDMECs stimulated with secretome from myofibroblasts differentiated under hyperglycemic conditions migrated significantly less

than those stimulated with secretome from differentiation under nornoglycemia. Intriguingly, the decreased migration in response to hyperglycemia was not associated with a concomitant decrease in VEGF-A, nor with an increase in TSP-1, at both the mRNA and protein level. In fact, VEGF-A mRNA expression was even significantly higher in HDMECs stimulated with myofibroblast secretome collected following hyperglycemic differentiation compared to normoglycemic differentiation, with no change at the protein level. This may suggest that other important angio-adaptive factors could be impacting the migratory activity of the endothelial cells. Moreover, given that the changes in VEGF-A mRNA are not seen at the protein level, there is a possibility of myofibroblasts differentiated under hyperglycemic conditions releasing factors that are impacting VEGF translation. Interestingly, there was not a decrease in phospho-p38 expression in the HDMECs treated with the conditioned media collected from hyperglycemic differentiation, despite the attenuation of migration observed in the Boyden chamber assay. This presents the possibility that other anti-angiogenic factors are interacting with the endothelial cells through mechanisms outside of the VEGF-R2 and p38 MAPK migration pathway.

We next looked at the impact of the same hyperglycemic conditions on the endothelial cells themselves. It is important to consider that if the progenitors or myofibroblasts exist in pathological conditions such as hyperglycemia, they will not be isolated. Rather, the endothelial cells themselves will be subjected to the same conditions. In normal physiological conditions, endothelial cells are exposed to blood glucose levels in the range of ~3.6-5.8 mM, and cells exposed to glucose levels greater than 10 mM, in vitro or in vivo as in diabetes mellitus, are considered be in a high

glucose condition (Popov, 2010). Type-2 diabetes is accompanied by a multitude of complications that negatively impact the integrity of the microvasculature and angiogenic activity (Beckman et al., 2002; Martin et al., 2003). High glucose levels disrupt endothelial cell homeostasis, leading to endothelial dysfunction and changes in both the macrovasculature and capillaries of the microvasculature (Popov, 2010). In diabetes, angiogenic responses are enhanced in some tissues (retina and kidney), while they are impaired in other tissues, leading to defective wound healing, peripheral circulation, and coronary circulation (Moriya & Ferrara, 2014). We chose to treat our HDMECs with 25mM glucose, as 20 mM to 30 mM glucose conditions have been widely used with endothelial cells to replicate hyperglycemia in culture (Gadad, Matthews, & Knott, 2013; Manna & Jain, 2014; Morigi et al., 1998; Popov, 2010; Syeda et al., 2012; Yang et al., 2008).

Hyperglycemic treatment had a dual impact on HDMEC migration, as it reduced basal migration under the no-stimulation condition, as well as the ability of the HDMECs to respond under the stimulatory condition. Interestingly, studies have pointed out both pro- and anti-migratory effects of hyperglycemic endothelial cells treatment (Huang et al., 2015; Wang, Yang, & Dong, 2013; Warren, Ziyad, Briot, Der, & Iruela-Arispe, 2014; Yu et al., 2006; Yuan et al., 2012). However, these works have mostly utilized Human Retinal Microvascular Endothelial Cells (HRMECs) or Human Umbilical Microvascular Endothelial Cells (HUVECs) with exposure to diabetogenic like conditions of high glucose over a relatively short 24 to 48 hour period. Few studies have specifically assessed HDMEC migration under following high glucose treatment. Gadad et al. reported a decrease in HDMEC migration analysed through wound healing assays

following 48 hours of high glucose treatment, while Syeda et al. have shown a decrease in HDMEC migration following 3-4 day exposure to high glucose (Gadad et al., 2013; Syeda et al., 2012). Our work looked to employ a “chronic” treatment of the HDMECs over 12 days, and 3 passages before assessing their migration.

We next looked to examine what intracellular changes the hyperglycemic exposure could be exerting on the endothelial cells that lead to the decreases in migration. We again measured VEGF-A and TSP-1 expression to gain an understanding of the autocrine angio-adaptive state of the cell. While there was an increase in TSP-1 mRNA expression, there was no change in HDMEC VEGF-A or TSP-1 protein levels, suggesting that these factors were not responsible for the changes seen in migration. With no change on the endogenous factor secretion, we chose to focus on the expression of p-VEGF-R2. There have been mixed reports regarding differences in p-VEGF-R2 expression levels in diabetic vs. non-diabetic individuals (Uchida & Haas, 2009). When we investigated p-VEGF-R2 in our HDMECs exposed to hyperglycemia, we found a 48% reduction in protein phosphorylation levels compared to cells under normoglycemic conditions. It has been suggested that excessive reactive oxygen species (ROS) produced by hyperglycemia promote activation of a noncanonical VEGF-R2 signalling pathway leading to increased endothelial cell VEGF-R2 internalization and degradation. As a result, traditional canonical ligand-dependent VEGF-R2 signalling is inhibited due to decreased availability of cell-surface VEGF-R2 (Moriya & Ferrara, 2014; Warren et al., 2014). Warren et al exposed HUVECs to high glucose for a minimum of 5 days, and found through wound healing assays that HUVECs cultured under high glucose migrated at a slower rate than cells under normal

glucose, under both basal conditions and in response to VEGF. These cells also had a 4 fold decrease in p-VEGF-R2 expression in response to VEGF stimulation, without changes in total VEGF-R2 protein. Cell surface biotinylation assays revealed HUVECs under high glucose had a decrease in surface abundance of VEGF-R2. They went on to show these HUVECs under high glucose exhibited increased ROS production (Warren et al., 2014). Interestingly, low concentrations of ROS can contribute to activating canonical (pro-angiogenic) signalling of VEGF-R2 under physiological conditions (Moriya & Ferrara, 2014). This could explain why in some cases, endothelial cells treated with high glucose have been shown to have increased migratory activity, as the exposure to the high glucose levels may not have been long enough to induce substantial ROS production.

It is enticing to hypothesize that increased ROS production is occurring in our HDMECs exposed to high glucose, leading to the decrease in p-VEGF-R2 expression, and subsequent loss of migratory ability. These results served as the first step in examining the myofibroblast relationship from the perspective of the endothelial cell. Decreased migration and reduced p-VEGF-R2 expression, possibly caused by increased ROS production, could also be indicative of changes in endothelial cell factor secretion.

Reactive oxygen species production in diabetic individuals is accompanied by increased production of Endothelin-1 by the dysfunctional endothelium (Creager & Libby, 2014; W. T. Wong, Wong, Tian, & Huang, 2010). In this context, Endothelin-1 is considered for its role as a vasoconstrictor and stimulator of vascular smooth muscle cell growth in the development of atherogenesis (Creager & Libby, 2014). Considering

that Endothelin-1 is a known stimulator of myofibroblast differentiation, this provides interesting avenues to explore in future works. Changes in the profile of secreted factors from the endothelial cells could significantly affect their relationship with the myofibroblast or their CD90+ progenitors.

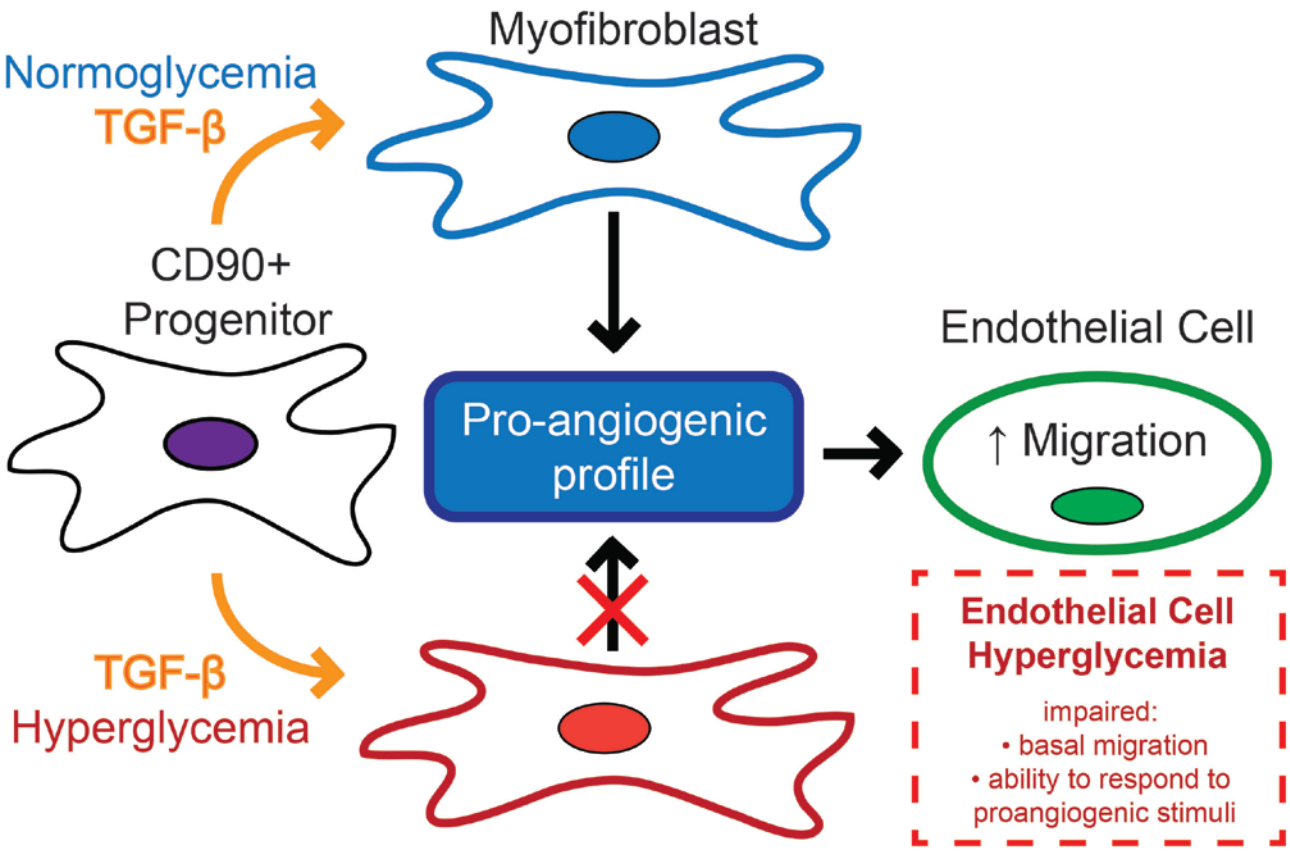

Figure 3. Summary of potential paracrine relationship between CD90+ progenitors, myofibroblasts, and endothelial cells, and how the presented relationship may be impacted by a pathological insult such as hyperglycemia.

A broad summary of our findings is illustrated in Figure 3. Through our results, it appears CD90+ skeletal muscle derived progenitors acquire a pro-angiogenic profile when differentiated into myofibroblasts. However, this pro-angiogenic profile is impaired when CD90+ progenitors are differentiated under hyperglycemia. Concurrently, hyperglycemia negatively impacts HDMVECs basal migration, and ability to respond to pro-migratory stimuli. Therefore, hyperglycemia may have a twofold impact in this potential paracrine relationship, as it impairs the ability of myofibroblasts to generate pro-angiogenic effectors, while at the same time impairing the ability of the endothelial cells to respond to those same factors.

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LIMITATIONS & FUTURE EXPERIMENTS

One of the major limitations of this study is that our human endothelial cells were from dermal origin, while our CD90+ progenitors were isolated from human skeletal muscle. Unfortunately, when beginning the project, we did not have access to human skeletal muscle endothelial cells. The popular choices in the literature for in-vitro primary human endothelial cell work are currently HUVECs, HDMECs, or human adipose microvascular endothelial cells (HAMECs). We felt that HDMECs were the most suitable choice to analyse the paracrine relationship between the cells, given the roles myofibroblasts play in dermal wound healing.

Dr. Grenier has since developed a method to isolate endothelial cells from human skeletal muscle. It would be interesting to perform the same experiments in this study with CD90+ progenitor cells and endothelial cells isolated from the same human skeletal muscle. Another interesting avenue would involve isolating endothelial cells or CD90+ progenitors from healthy patients and diabetic patients, and examining the paracrine relationship if the cells maintain their pathological phenotype in vitro.

Other future experiments involve exploring this relationship from the perspective of the endothelial cell and the impact it could have on progenitor cell differentiation, or myofibroblast survival and functionality. This would involve collecting conditioned media from endothelial cells cultured under normal and high glucose conditions, and treating progenitor cells and differentiated myofibroblasts, while monitoring changes in α-smooth muscle actin, collagen production, and contractility. If changes are seen in progenitor or differentiated myofibroblasts, the conditioned media itself could be analyzed for pro-

differentiation factors such as endothelin-1, sphingosine-1-phosphate, or TGF-β. These experiments would help “complete the loop” by establishing the reciprocal paracrine relationship from the perspective of both cell types.

Further work could be done to examine the possible bio-physical relationship between the cell types by utilizing co-culture techniques. The viability and functionality of both cell types could be monitored with by co-culturing the cells with different concentrations in respect to one another. Tube formation assays using both cell types concurrently could provide valuable information about the functional impact of the physical relationship on both cell types.

Lastly, more work could be done to establish the anatomical presence of the progenitors, and how it may change once differentiation takes place. Histological work in samples from healthy or diabetic individuals could provide useful information to the quantity and location of these progenitors or differentiated myofibroblasts. Being able to show that the cells are in the proximity of the microvasculature strengthens the results examining their potential paracrine relationship.

CONCLUSION

Through this project, we have been able to provide preliminary evidence aimed at answering the broad questions we had identified regarding the physiological roles of mesenchymal stem cells, and myofibroblasts in angiogenesis. We have focused on the possible role of a supporting cell type other then the muscle tissue itself in skeletal muscle angiogenesis. Concurrently, we have expanded on the characterization of a newly identified skeletal muscle myofibroblast progenitor by examining its potential physiological role.

We have shown that myofibroblasts acquire a pro-angiogenic profile following differentiation from CD90+ progenitor cells derived from human skeletal muscle. This was demonstrated with good correspondence between changes seen at the molecular level, and changes in cell functionality. Interestingly, this pro-angiogenic profile seems to be negatively altered when differentiation takes place under hyperglycemic conditions. Through these results, we believe we have established a good basis for the relationship between the CD90+ progenitor, myofibroblast, and endothelial cells.

These results focus on the relationship from the perspective of the myofibroblast, and its actions on the endothelial cell. Through future experiments, we would like to explore the relationships from the perspective of the endothelial cell. If changes in myofibroblast differentiation are seen through altered secreted factors from endothelial cells under hyperglycemia, then we can begin to suggest that there is in fact a reciprocal relationship that is negatively impacted in a metabolically disturbed state.

FIGURES

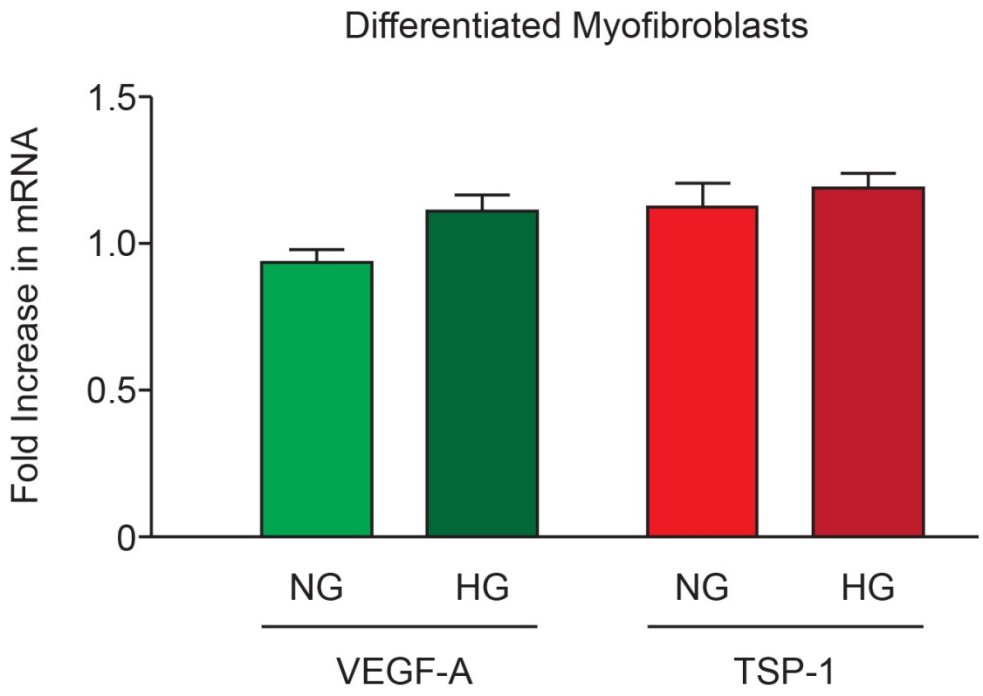
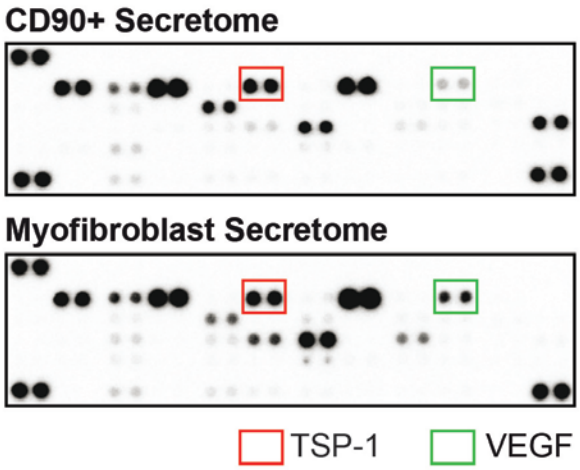


Figure 4. Myofibroblast VEGF-A and TSP-1 mRNA expression. CD90+ progenitor cells were differentiated into myofibroblasts under normoglycemic (5 mM glucose) or hyperglycemic (25 mM glucose) conditions. VEGF-A and TSP-1 mRNA levels were measured by Tapman qPCR and relative expression quantified relative to the housekeeping gene HPRT. Data are shown as means ± SEM with n = 4 groups per condition.

A



B

Pro-angiogenic	CD90+	Myofib
Angiogenin	100	213
Ang-1	100	310
PDGF-AA	100	750
VEGF	100	237
Anti-angiogenic		
Activin-A	100	122
Pentraxin-3	100	49
TIMP-1	100	87
TSP-1	100	99

Figure 5. Myofibroblast secretome is enriched in pro-angiogenic factors. The expression level of 55 proteins involved in regulating angio-adaption was measured in CD90+ progenitor and differentiated myofibroblast secretomes using an angiogenesis proteome profiler array. (A) Representative proteome profiler membranes of secreted proteins from CD90+ precursors or differentiated myofibroblasts. (B) Relative changes in established pro-angiogenic and anti-angiogenic factors.

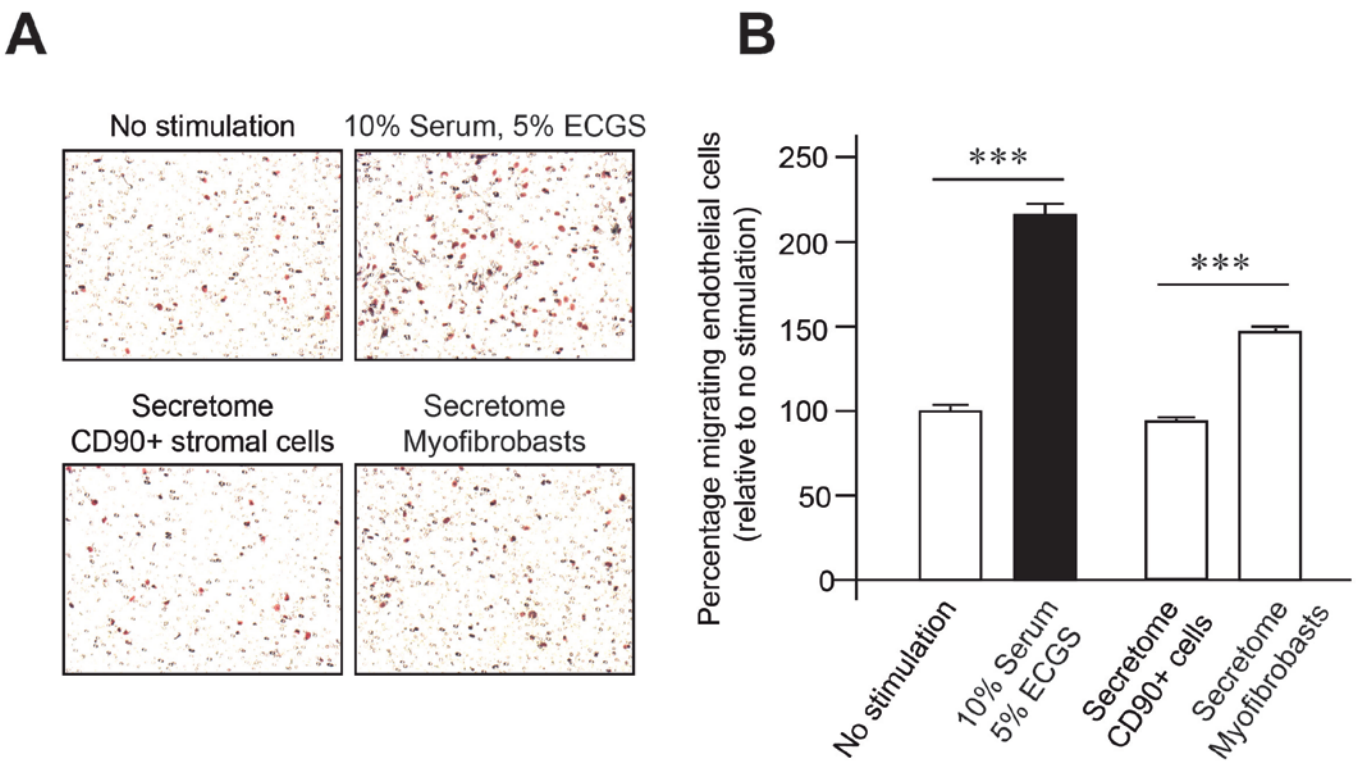


Figure 6. Myofibroblast secretome stimulates HDMEC migration. Representative Boyden Chamber migration assay from primary endothelial cells stimulated with CD90+ progenitor or differentiated myofibroblast secretome. (A) Representative photos of the migration of endothelial cells in the Boyden Chamber, in vitro model. Cells are allowed to migrate for 4.5 hours through a nitrocellulose membrane with 8 micron pores, and are then stained with Giemsa. **(B)** Quantification of the migration shown in (A). Values shown are averages obtained with SEM from 3 independent experiments, each comprising n = 6 wells per condition migration. Four counts per well were completed. 10% serum, 5% ECGS served as positive control. Significantly different within each condition, ***, $P \leq 0.001$.

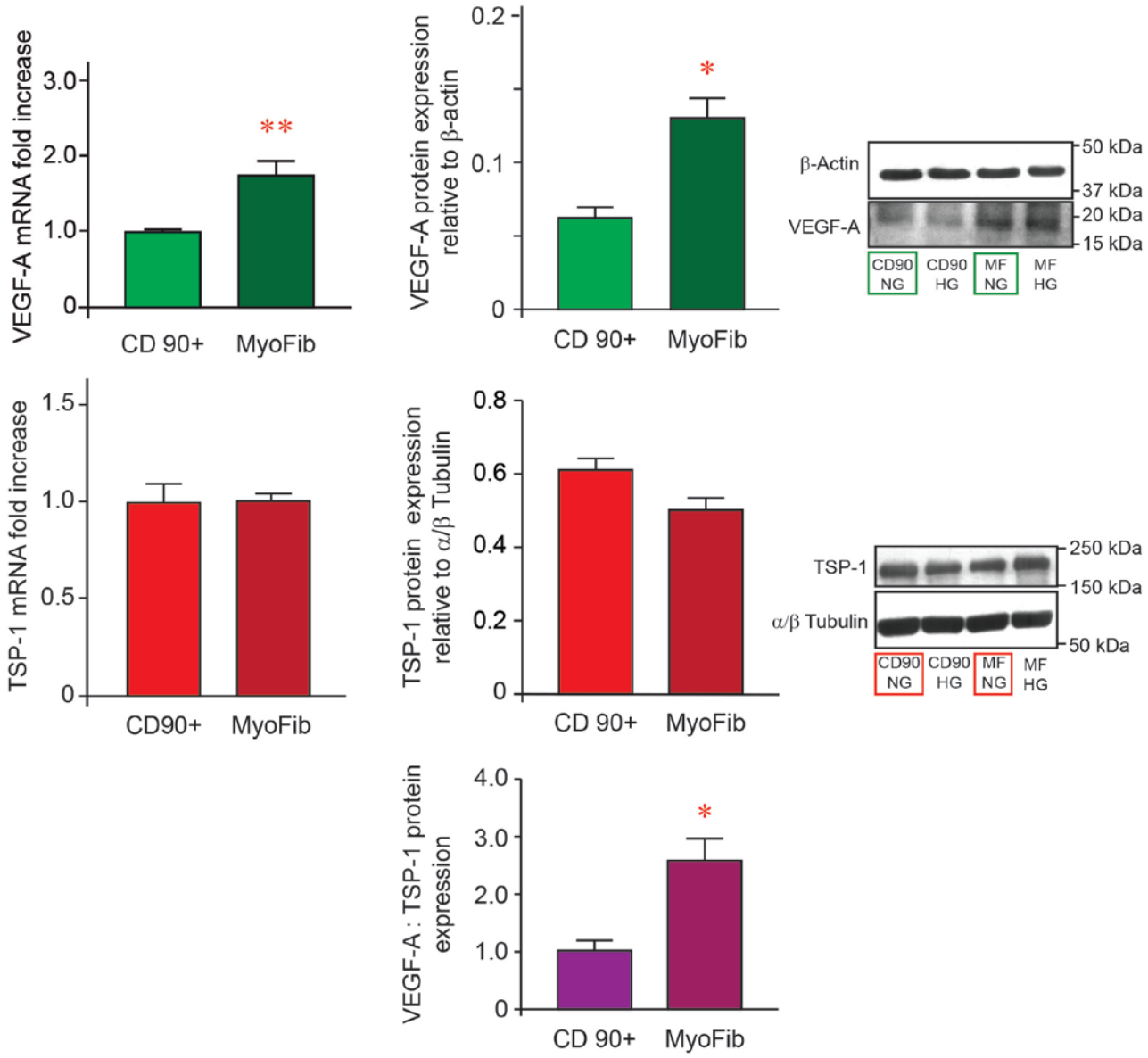


Figure 7. VEGF-A and TSP-1 mRNA and protein expression in HDMEC treated with CD90+ progenitor or myofibroblast secretome. HDMECs were stimulated with secretome from CD90+ and differentiated myofibroblasts for 16 hours. VEGF-A and TSP-1 mRNA levels were measured using Taqman qPCR and relative expression quantified relative to the housekeeping gene HPRT. Respective protein levels were measured using western blot, and further analysed as a VEGF to TSP-1 ratio. Data are shown as mean ± SEM from n = 3 samples from each differentiation condition. Significantly different from CD90+: ** $P \leq 0.01$; * $P \leq 0.05$.

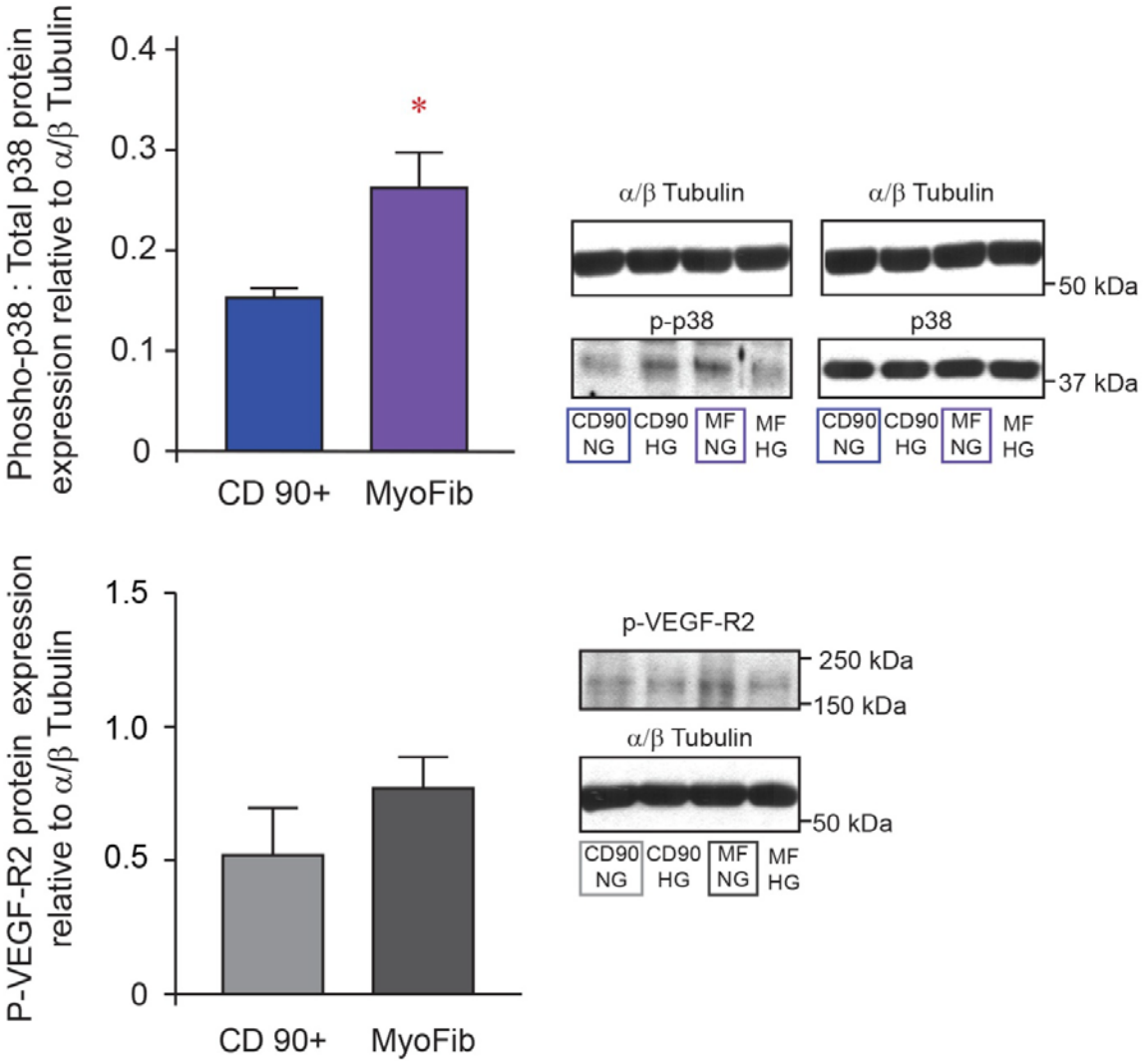
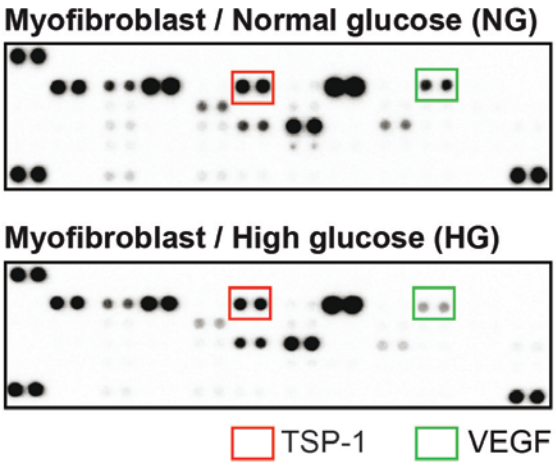


Figure 8. p38, phospho-p38, and phospho-VEGF-R2 protein expression in HDMECs treated with CD90+ progenitor or myofibroblast secretome. HDMECs were stimulated with secretome from CD90+ and differentiated myofibroblasts for 16 hours. p-VEGF-R2, p38, and p-p38 protein levels were measured using western blot. Data are shown as mean ± SEM from n = 3 samples from each differentiation condition. Significantly different from CD90+: *, $P \leq 0.05$.

A



B

Pro-angiogenic	NG	HG
Ang-1	100	60
HB-EGF	100	21
PDGF-AA	100	68
VEGF	100	56
Anti-angiogenic		
Angiostatin	100	312
Serpin-F1	100	93
TIMP-1	100	83
TSP-1	100	79

Figure 9. Hyperglycemic differentiation inhibits the pro-angiogenic profile of myofibroblast secretome. The expression level of 55 proteins involved in regulating angio-adaption was measured in differentiated myofibroblast secretomes using an angiogenesis proteome profiler array. **(A)** Representative proteome profiler membranes of secreted proteins from myofibroblasts differentiated under normo- or hyperglycemia. **(B)** Relative changes in established pro-angiogenic and anti-angiogenic factors.

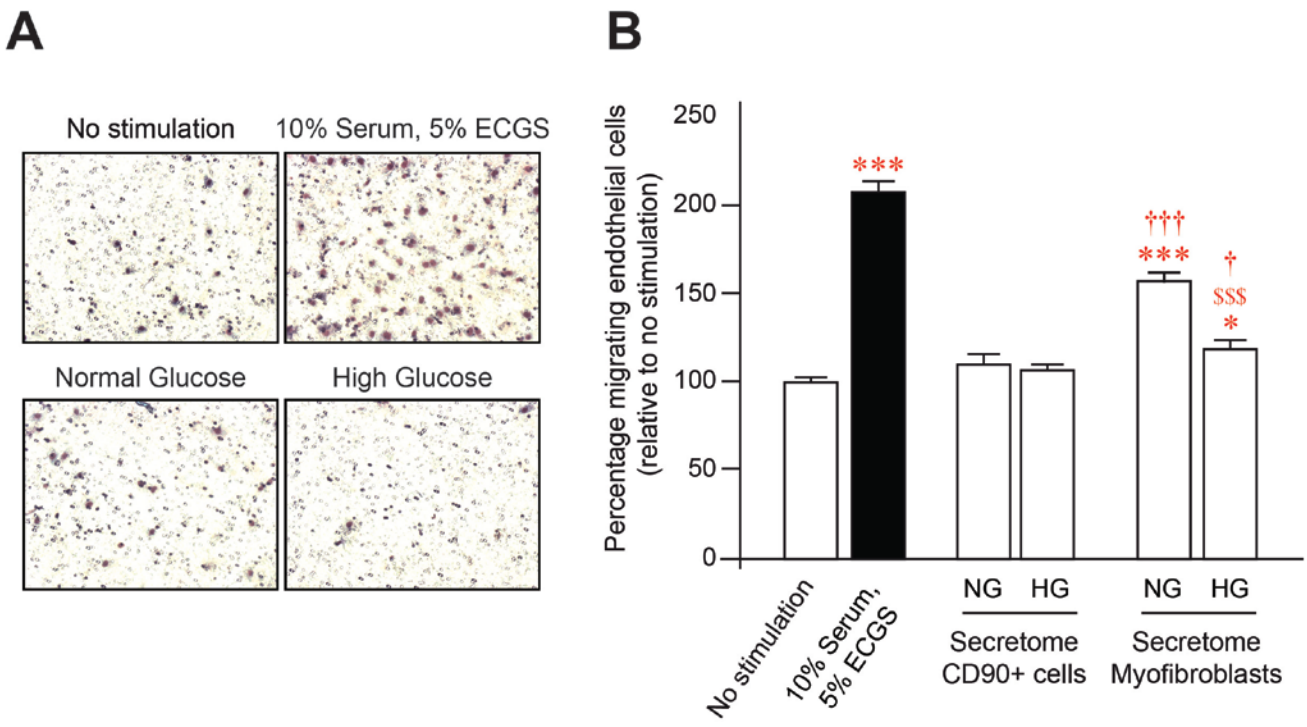


Figure 10. Myofibroblast secretome obtained following hyperglycemic differentiation reduced HDMEC migration. (A) Representative Boyden Chamber migration assays from primary endothelial cells stimulated with CD90+ and myofibroblast secretomes obtained under normoglycemic (NG) or hyperglycemic (HG) differentiation. Cells are allowed to migrate for 4.5 hours through a nitrocellulose membrane with 8 micron pores, and are then stained with Giemsa. 10% serum, 5% ECGS served as positive control. **(B)** Quantification of the migration shown in (A). Values shown are averages obtained with SEM from 3 independent experiments, each comprising n = 6 wells per condition migration. Four counts per well were completed. Significantly different from no stimulation: ***, $P \leq 0.001$; *, $P \leq 0.05$ (1W-ANOVA).

2W-ANOVA analysis of the effect of differentiation and HG on the ability of the secretome to stimulate migration: Effect of differentiation, †††, $P \leq 0.001$; †, $P \leq 0.05$. Effect of HG, $$$ $P \leq 0.001$.

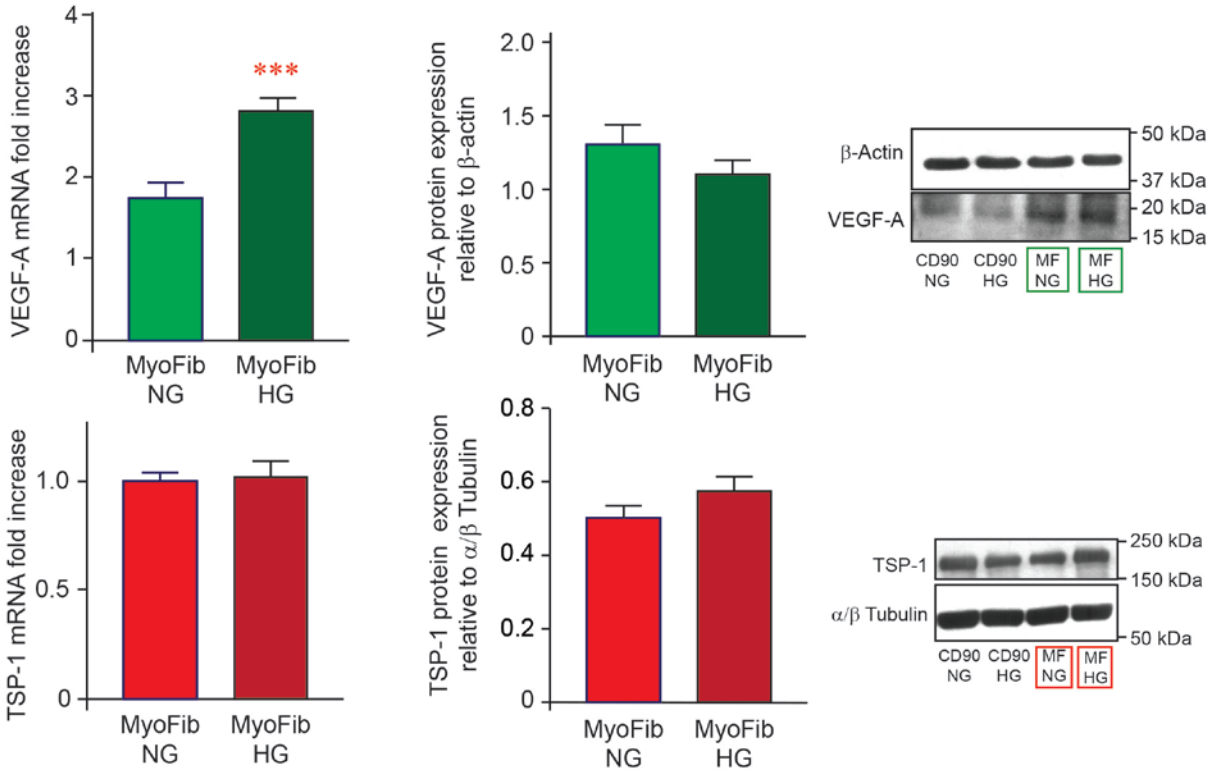


Figure 11. VEGF-A and TSP-1 mRNA and protein expression in HDMECs treated with secretome from myofibroblasts differentiated under normo- or hyperglycemia. HDMECs were stimulated for 16 hours with secretome from myofibroblasts differentiated under normo- or hyperglycemic conditions. VEGF-A and TSP-1 mRNA levels were measured using Taqman qPCR, and relative expression quantified relative to the housekeeping gene HPRT. Respective protein levels were measured using western blot. Data are shown as mean ± SEM from n = 3 samples from each differentiation condition. Significantly different from MyoFib NG: *** $P \leq 0.001$.

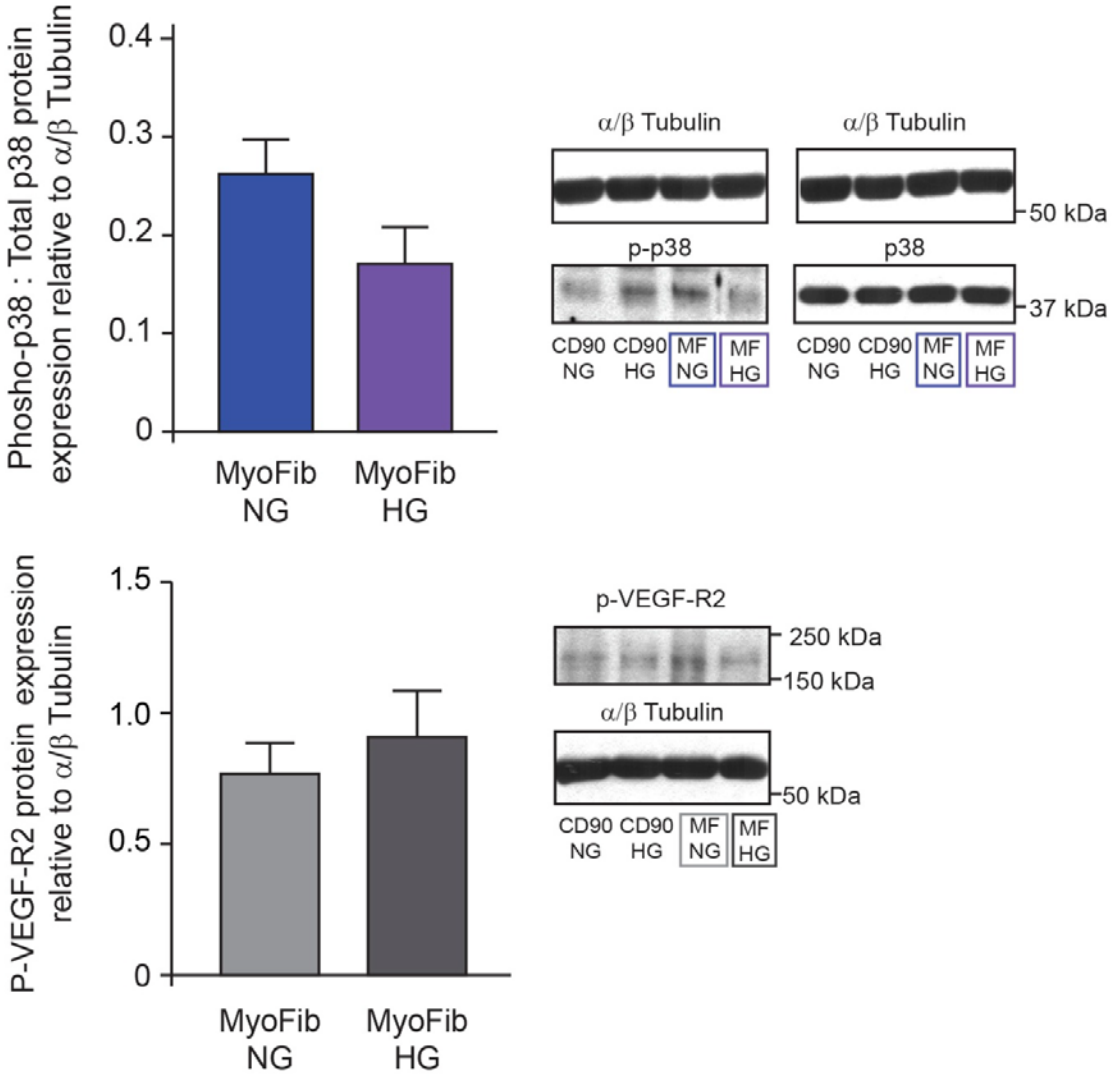


Figure 12. p38, phospho-p38, and p-VEGF-R2 protein expression in HDMEC treated with secretome from myofibroblasts differentiated under normo- or hyperglycemia. HDMECs were stimulated for 16 hours with secretome from myofibroblasts differentiated under normo- or hyperglycemic conditions. P-VEGF-R2, p38, and p-p38 protein levels were measured using western blot. Data are shown as mean ± SEM from n = 3 samples from each differentiation condition.

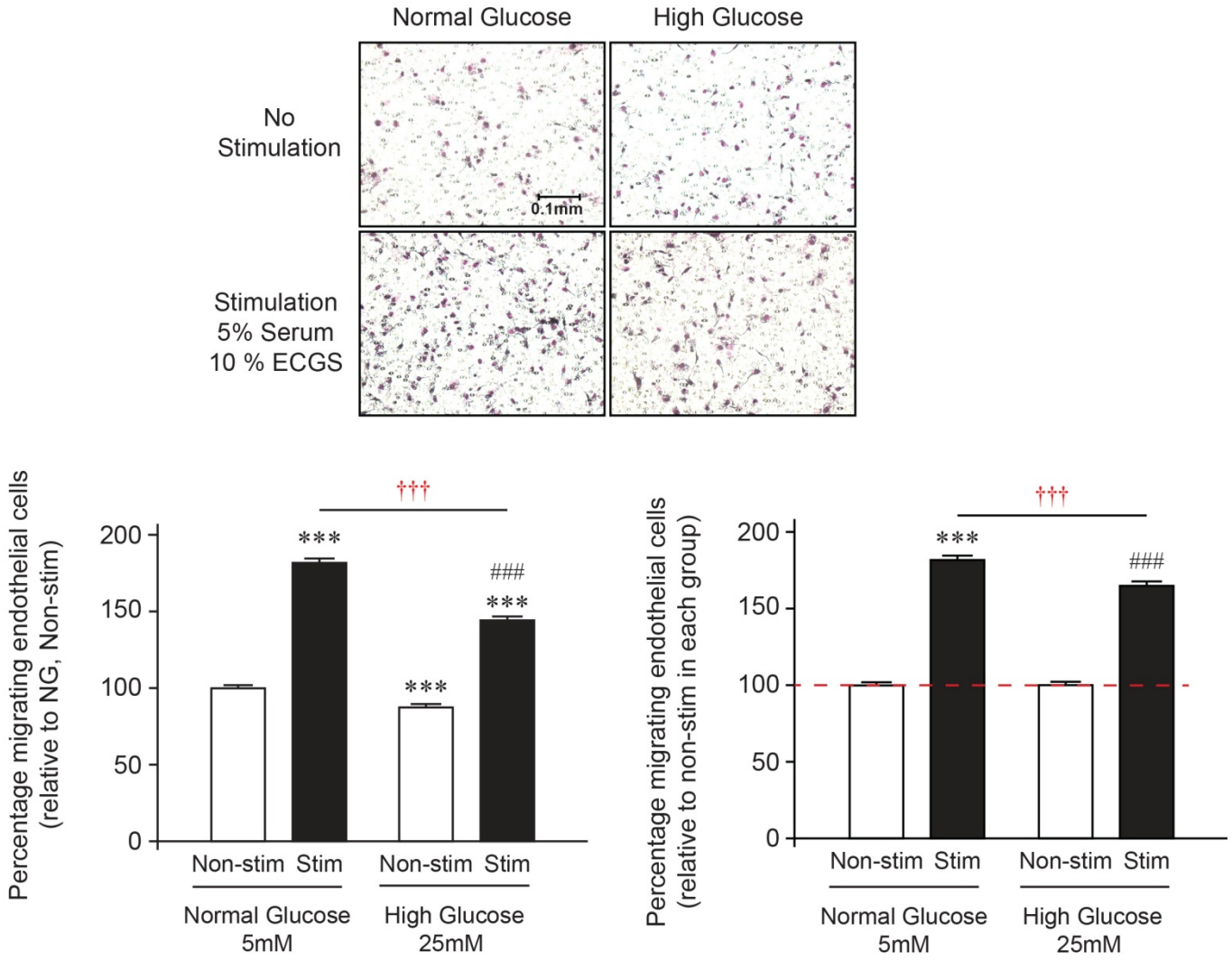


Figure 13. Inhibitory effect of chronic high glucose on the migratory activity of primary human microvascular endothelial cells. Primary human microvascular endothelial cells were grown under normoglycemic (5mM) or hyperglycemic (25mM) conditions over 12 days (3 passages, p5 to p8). **(A)** Representative photos of the migration of endothelial cells in the Boyden Chamber, in vitro model. Cells are allowed to migrate for 4.5 hours through a nitrocellulose membrane with 8 micron pores, and are then stained with Giemsa. **(B)** Quantification of the migration shown in (A). Values shown are averages obtained with SEM from 3 independent experiments, each comprising n = 6 wells per condition migration. Four counts per well were completed. Significantly different from no stimulation, normal glucose, ***, $P \leq 0.001$. Significantly different from no stimulation, high glucose ###, $P \leq 0.001$. Significantly different from normal glucose, stimulation, †††, $P \leq 0.001$.

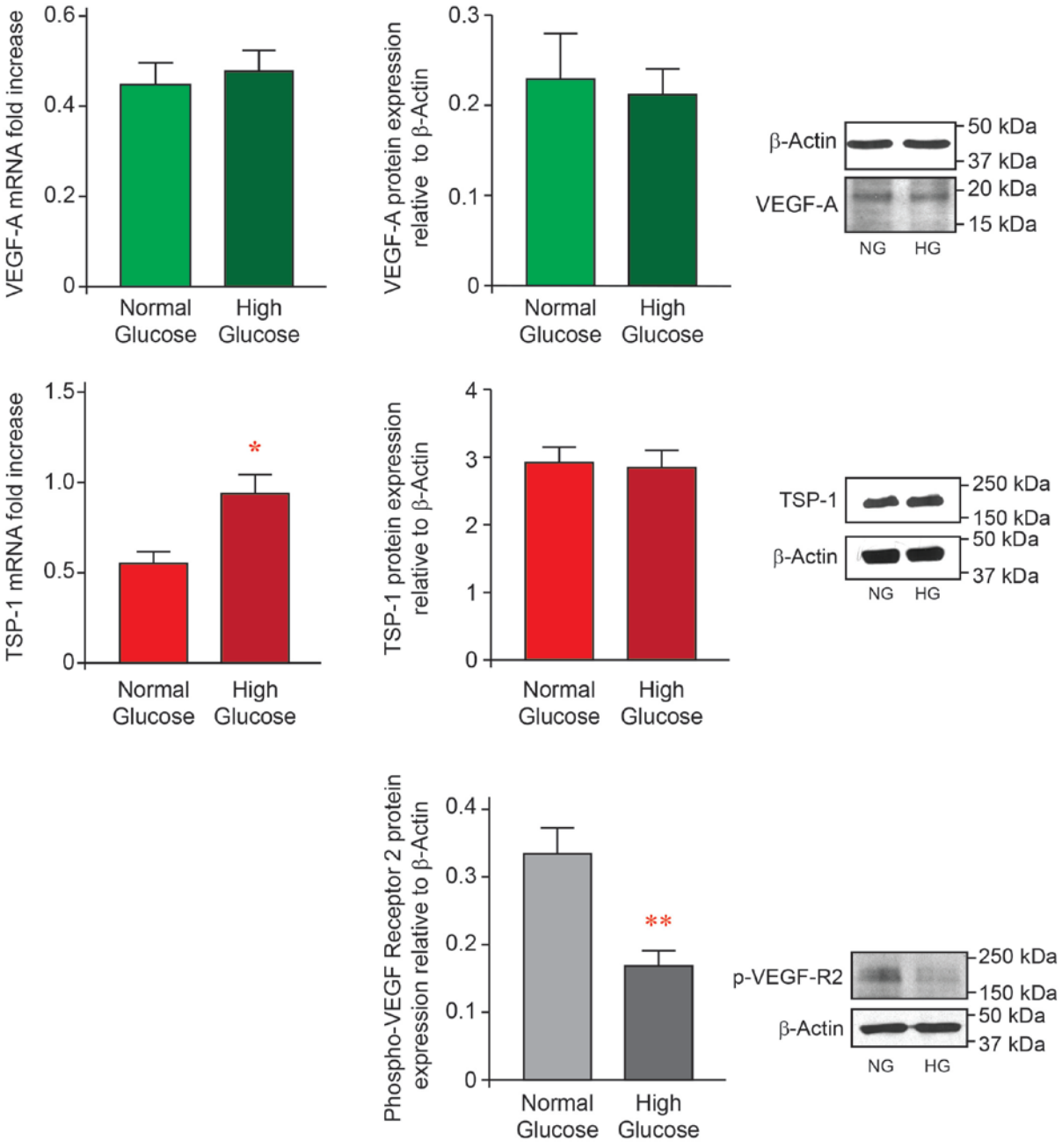


Figure 14. VEGF-A, TSP-1 mRNA and VEGF-A, TSP-1, and p-VEGF-R2 protein expression in HDMEC cultured under normo- or hyperglycemia. HDMECs were cultured for 12 days (3 passages) under normoglycemic (5 mM glucose) or hyperglycemic (25 mM glucose) conditions. VEGF-A and TSP-1 mRNA levels were measured using Taqman qPCR, and relative expression quantified relative to the housekeeping gene HPRT. VEGF-A, TSP-1, and p-VEGF-R2 protein levels were measured using western blot. Data are shown as mean ± SEM from n = 4 samples from each differentiation condition. Significantly different from Normal Glucose: *, $P \leq 0.05$, ** $P \leq 0.01$.

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APPENDICES

APPENDIX A – Western Blotting

Percentage of SDS gel, electrophoresis time, transfer time, and antibody concentration used during western blotting.

Protein Target	Percentage Acrylamide Gel	Electrophoresis Time (minutes)	Electrotransfer Time (minutes)	Primary antibody conc.
p38	10	70	70	1:1000
p-p38	10	70	70	1:500
p-VEGF-R2	7.5	55	75	1:1000
TSP-1	7.5	60	70	1:200
VEGF-A	15	65	70	1:500

Loading Controls	
Protein Target	**Primary Antibody Concentration**
α/β Tubulin	1:1000
β-Actin	1:4000

Western Blot Solutions

Separating Buffer				
1.5 M Tris, pH 8.8 + 0.4% SDS				
Total Volume	**Molecular Weight**	**Final Conc.**	**50 ml**	**100 ml**
Tris Base	121.14 g/mol	1.5 M	9.09g	18.18g
20% SDS	288.38 g/mol	0.4%	1 ml	2 ml
H2O	N/A	N/A	Up to final volume	
pH	pH 8.8			
NOTE: STORE AT 4°C				

Stacking Buffer				
0.5 M Tris, pH 6.8 + 0.4% SDS				
Total Volume	**Molecular Weight**	**Final Conc.**	**50 ml**	**100 ml**
Tris Base	121.14 g/mol	0.5 M	3.03g	6.06g
20% SDS	288.38 g/mol	0.4%	1 ml	2 ml
H2O	N/A	N/A	Up to final volume	
pH	pH 6.8			
NOTE: STORE AT 4°C				

5X Running/Electrophoresis Buffer				
Total Volume	**Molecular Weight**	**Final Conc. (5X)**	**Final Conc. (1X)**	**1L (5X)**
Tris	121.14 g/mol	125 mM	25 mM	15.15g
Glycine	75.07 g/mol	960 mM	192 mM	72.00g
SDS	288.38 g/mol	17.34 mM	3.47 mM	5.00g
H2O	N/A	N/A	Up to Final Volume	
NOTE: STORE AT R.T.				

5X Transfer Buffer W/O Methanol				
Total Volume	**Molecular Weight**	**Final Conc. (5X)**	**Final Conc. (1X)**	**1L (5X)**
Tris	121.14 g/mol	125 mM	25 mM	15.15g
Glycine	75.07 g/mol	960 mM	192 mM	72.00g
H2O	N/A	N/A	Up to Final Volume	
NOTE: STORE AT R.T.				
1X TRANSFER BUFFER SHOULD HAVE 20% METHANOL WHEN COMPLETE				

10x Tris Solution				
Total Volume	**Molecular Weight**	**Final Conc.**	**500 ml**	**1L**
Tris	121.14 g/mol	500 mM	30.275g	60.55g
pH	pH 7.6			
H2O	N/A	N/A	Up to Final Volume	
NOTE: STORE AT R.T.				

Denaturing Blue Buffer			
Compound	**Molecular Weight (g/mol)**	**Final Concentration**	**For 10 ml final volume**
Sucrose	342.30	2.69 mM	0.92 g
SDS	288.38	0.28 M	0.80 g
Beta-Mercaptoethanol	78.13	2.84 M	2 mL
Bromophenol Blue	691.94	14 µM	10 mg
0.5 M Tris pH 6.8	N/A	N/A	2 mL
Water	N/A	N/A	Up to final volume
Stored at -20°C			

5% / 5% BSA in 0.1% TTBS	
Milk/BSA	5% of final volume in weight (grams)
0.1% TTBS	Up to final volume
Stored at 4°C	

APPENDIX B

DMEM vs. RPMI – Myofibroblast differentiation media

Myofibroblast ability to stimulate HDMEC migration is affected by the type differentiation media utilized. Using Roswell Park Memorial Institute (RPMI) medium, there is a 64% increase in HDMEC migration following stimulation with myofibroblast conditioned media compared to CD90+ progenitor conditioned media. However, when using Dulbecco's Modified Eagles Medium (DMEM), there is no change in the ability of the conditioned media to stimulate HDMEC migration [Figure A1]. Therefore, the media itself appears to have significant impact on the secreted factors from the differentiated myofibroblast. This poses questions to be answered in future experiments, specifically, identifying which specific factors could be responsible for the changes in myofibroblast secretion. Ultimately this represents one of the limits with in vitro cell work, as deciding which medium is a better representation of in-vivo conditions is a factor to be taken into consideration.

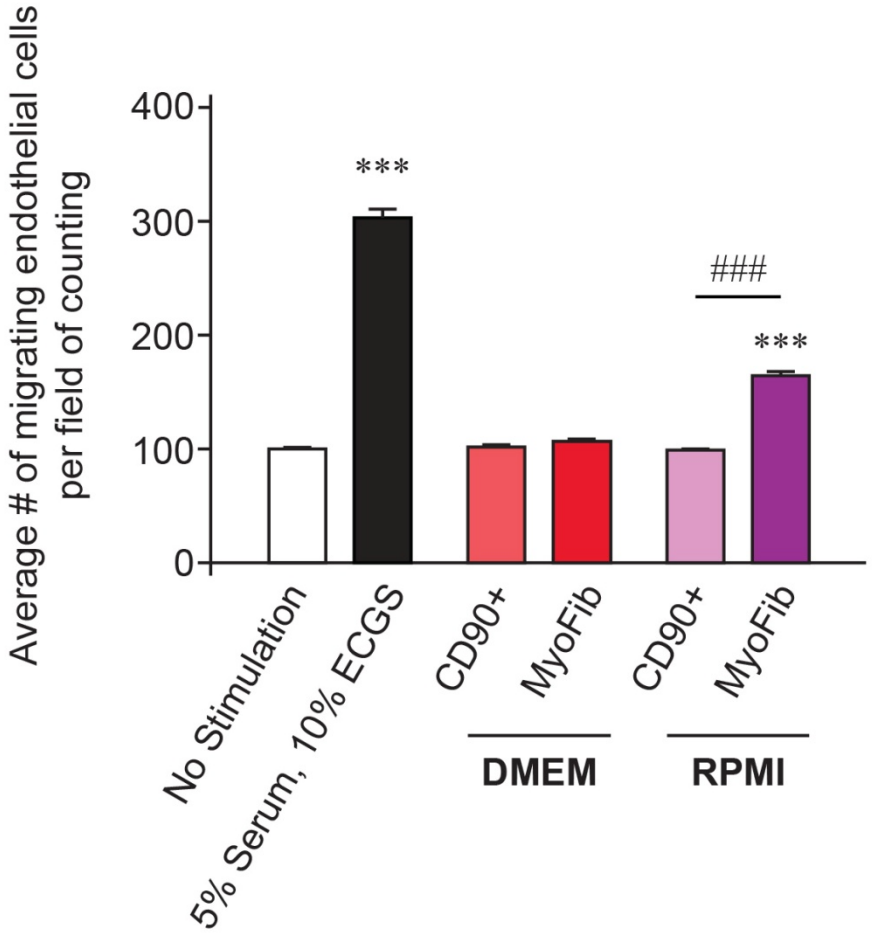

Figure A1. Myofibroblast secretome obtained following differentiation using DMEM does not stimulate HDMEC migration. Quantification of Boyden Chamber migration assays from primary human dermal endothelial cells stimulated with CD90+ and myofibroblast secretome obtained under differentiation and conditioning with DMEM or RPMI**.** Values shown are averages obtained with SEM from 3 independent experiments, each comprising n = 4 wells per condition migration. Four counts per well were completed. Significantly different from no stimulation, ***, $P \leq 0.001$. Significantly different from CD90+, ###, $P \leq 0.001$.

APPENDIX C

Side projects and contributions to the lab outside of my thesis project.

Calibration and validation of the Boyden Chamber migration assay.

Prior to my arrival in the lab, the Boyden Chamber migration assay was not a routinely used experiment. Therefore, when we acquired the assay, my first task involved the calibration and validation of the assay with our dermal and adipose microvascular endothelial cells. Calibrating the assay involved the adjustment of variables including pore size selection, cell suspension concentration, positive control selection, and ideal migration time. The size of the pore in the nitrocellulose membrane must be matched to the specific cell type being analysed. A pore that is too large will allow any cell with a basal migratory activity to move easily through the membrane, and differences in migration will not be readily detectable. An appropriate cell suspension density to be loaded into the upper chamber of each well also had to be established. A cell density of 440 cells/µl allowed for an appropriate migration under various conditions. Most importantly, consistent and reproducible positive control conditions had to be established. This was done by using ECM with increasing amounts of fetal bovine serum and endothelial cell growth supplement. An ideal solution was found by using ECM supplemented with 10% FBS, and 5% ECGS, which consistently provided an 80 – 100 % increase in cell migration, while ECM with 1% serum served as the no stimulation condition.

Assessing Mdm2 phosphorylation on Serine 166 as a key regulator of Human Dermal Microvascular Endothelial Cells

My first project involved utilising the Boyden Chamber assay to examine the role of Mdm2 signalling in cell migration. Prior in vivo and in vitro work from the lab had identified Mdm2 as a novel regulator of angiogenesis (Roudier et al., 2013; Roudier, Forn, Perry, & Birot, 2012). This previous work in the lab examined this relationship form a molecular pathway perspective, measuring changes in pro- and anti-angiogenic factors, and downstream signalling changes. The aim of my experiments was to examine this relationship from an in vitro cell functionality perspective.

Primary human microvascular endothelial cells were infected with lentivirus encoding Mdm2 (Mdm2 Wild-type) or a mimetic of the phosphor-activated form, through phosphorylation of serine residue 166 (p-Ser166-Phospho-mimetic Mdm2). Both WT-Mdm2 and S166D-Mdm2 showed increased protein expression levels of Mdm2 vs. non-infected HDMEC (281% and 191%, respectively) (Aiken & Roudier, manuscript in preparation)**.** The migratory activity of HDMEC was evaluated in the Boyden chamber assay [Figure A2]. Over-expression of WT-Mdm2 did not affect basal HDMEC migration [Figure A2, B, Non-infected HDMEC: 100.0 ± 2.3 vs. WT-Mdm2: 95.0 ± 3.4, $P>0.05$]. In contrast, over-expression of S166D-Mdm2 significantly enhanced the basal migration level by 38% [Figure A2, B, Non-infected HDMEC: 100.0 ± 2.3 vs. S166D-Mdm2: 138.0% ± 8.4, $P\leq0.001$]. Stimulation with 10% FBS and 5% ECGS enhanced migration in all cell lines, respectively by 95% (±8.76) for Non-infected HDMEC, 116% (±4.76) in WT-Mdm2, and 275% (±4.17) for S166D-Mdm2 compared to each corresponding non-stimulated condition [Figure A2, B, $P\leq0.001$]. The percentage of HDMEC migrating

under FBS/ECGS stimulation was significantly higher in the S166D-Mdm2 when compared to both stimulated Non-infected and WT-Mdm2 cells [Figure A2, B, $P \leq 0.001$). This suggests that Mdm2 phosphorylation on serine 166 is important for basal and stimulated primary microvascular endothelial cell migration.

Montpellier – Skeletal Muscle and Adipose Tissue Capillarization, and VEGF-A/TSP-1 mRNA expression.

My other main contribution to the lab outside of my thesis was through an ongoing collaborative project with the Université of Montpellier. Skeletal muscle (vastus lateralis) and adipose tissue samples were collected and sectioned from 31 post-menopusal women. RNA extract was also collected from the same tissues. Insulin status was defined using the homeostatic model assessment, HOMA. Ten women were healthy and served as controls (BMI<25 Kg/m^2, HOMA<3), while 12 were obese and insulin sensitive (BMI>30 Kg/m^2, HOMA<3), and 9 were obsess and insulin resistant (BMI>30 Kg/m^2, HOMA>3).

Previous work in the lab had examined the capillary to fiber ratio, the capillary density, and the adipocyte cross sectional area in a number of the sectioned adipose tissue samples. This was accomplished through immunohistochemical staining of capillaries by targeting CD31. My role in the project was to complete the immunohistochemical staining of additional adipose samples, and to begin the staining of muscle tissue samples, while also completing qPCR measurements of angio-adaptive factors in the RNA extracts from the same patients.

There were no significant differences in muscle tissue capillary density [Figure A3, 346.1 ± 15.6 cap/mm^2 Ctrl vs. 295 ± 14.7 cap/mm^2 Ob-IS vs. 315.3 ± 30.1 cap/mm2 Ob-IR] or fiber cross sectional area [Figure A3, 3991 ± 229 μm^2 Ctrl vs. 4145 ± 296 μm^2 Ob-IS vs. 3480 ± 549 μm^2 Ob-IR] between the groups. However, capillary to fiber ratio was 11% lower in obese insulin sensitive subjects [Figure A3, 1.36 ± 0.05 C/F Ctrl vs. 1.22 ± 0.04 C/F Ob-IS,*, $P \leq 0.05$] and 27% lower in obese insulin resistant subjects [Figure A3, 1.36 ± 0.05 C/F Ctrl vs. 0.99 ± 0.03 C/F Ob-IR, ***, $P \leq 0.001$] compared to healthy controls. When focusing on the two obese groups alone, there is an 18% decrease in capillary to fiber ratio between insulin sensitive and insulin resistant subjects [Figure A3, 1.22 ± 0.04 C/F Ob-IS vs. 0.99 ± 0.03 C/F Ob-IR, †††, $P \leq 0.001$].

There was no significant change in VEGF-A and TSP-1 mRNA expression between the groups in either the muscle or adipose tissue samples [Figure A4].

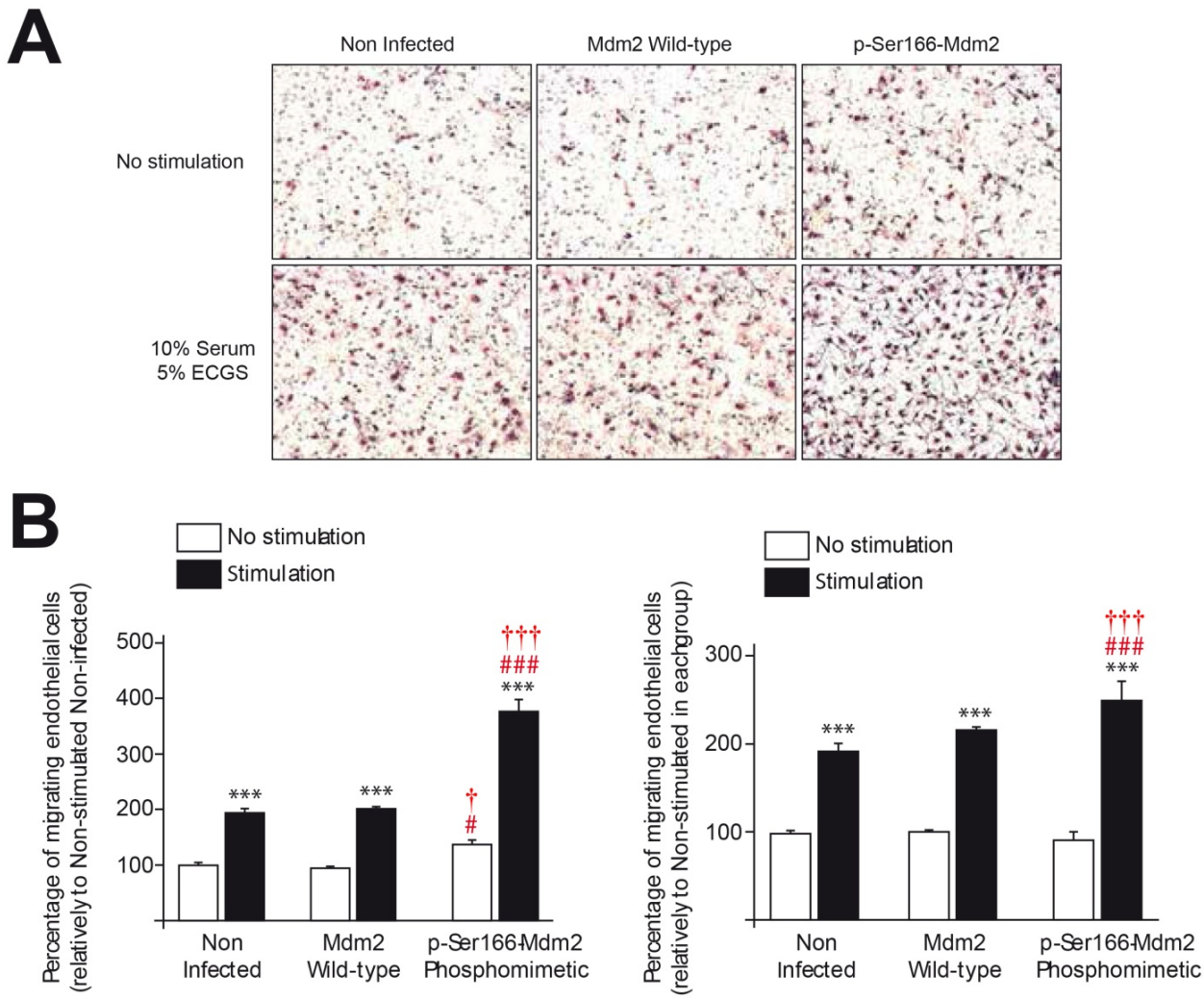

Figure A2. The effect of Mdm2 phosphorylation on serine 166, as a regulator of endothelial cell migration. (A) Representative Boyden Chamber migration assay from primary endothelial cells infected with lentivirus encoding Mdm2 (Mdm2 Wild-type) or a mimetic of the phosphor-activated form, through phosphorylation of serine residue 166 (p-Ser166-Phospho-mimetic Mdm2). Cells were allowed to migrate for 4.5 hours through a nitrocellulose membrane with 8 micron pores, and are then stained with Giemsa. **(B)** Quantification from 2 independent experiments, each compromising n = 6 per condition migration. Four counts were completed per well. Un-supplemented ECM served as “no stimulation” condition, while ECM supplemented with 10% serum, 5% ECGS served as “stimulation” condition.

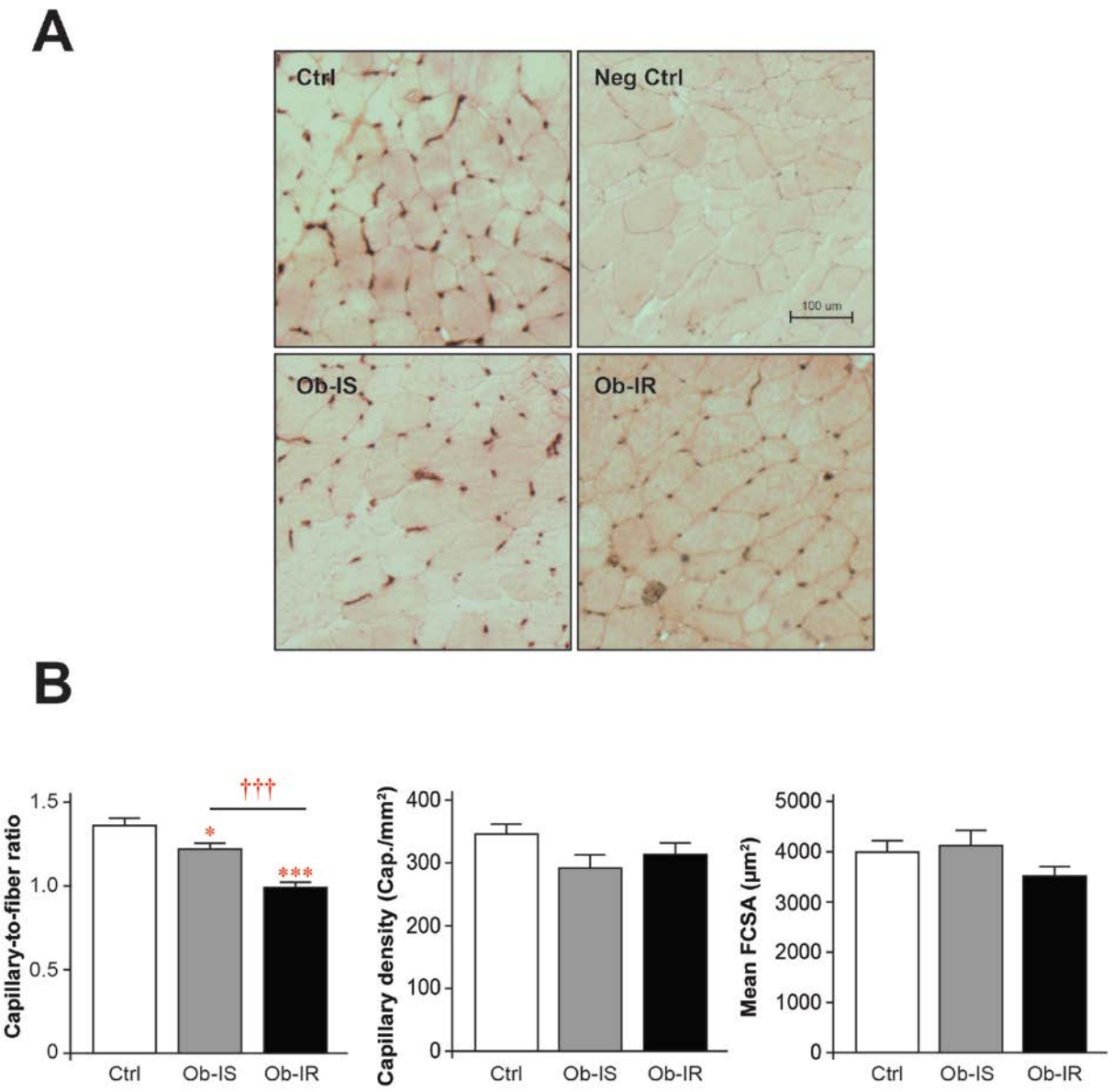

Figure A3. Effect of obesity and insulin resistance on human skeletal muscle capillarization. (A) Representative pictures of cryosections of human vastus lateralis muscles stained for the endothelial marker CD31. Muscle biopsies were obtained from healthy control female subjects (Ctrl, n = 7, BMI<25 Kg/m^2, HOMA<3), obese insulin sensitive (Ob-IS, n = 11, BMI>30 Kg/m^2, HOMA<3) and obese insulin resistant (Ob-IS, n = 11, BMI>30 Kg/m^2, HOMA<3) female patients. Scale bar, 100 µm. (**B)** Capillaries and fibers were counted on an average of 4-6 pictures per subject in order to determine capillary-to-fiber ratio, the capillary density (cap/mm^2), and the mean cross sectional fiber area (FCSA, µm^2). Data are represented as mean ± SEM. Significantly different from Control: ***, *P*≤0.001, *, *P*≤0.05. Significant difference between Ob-IS and Ob-IR: †††, *P*≤0.001.

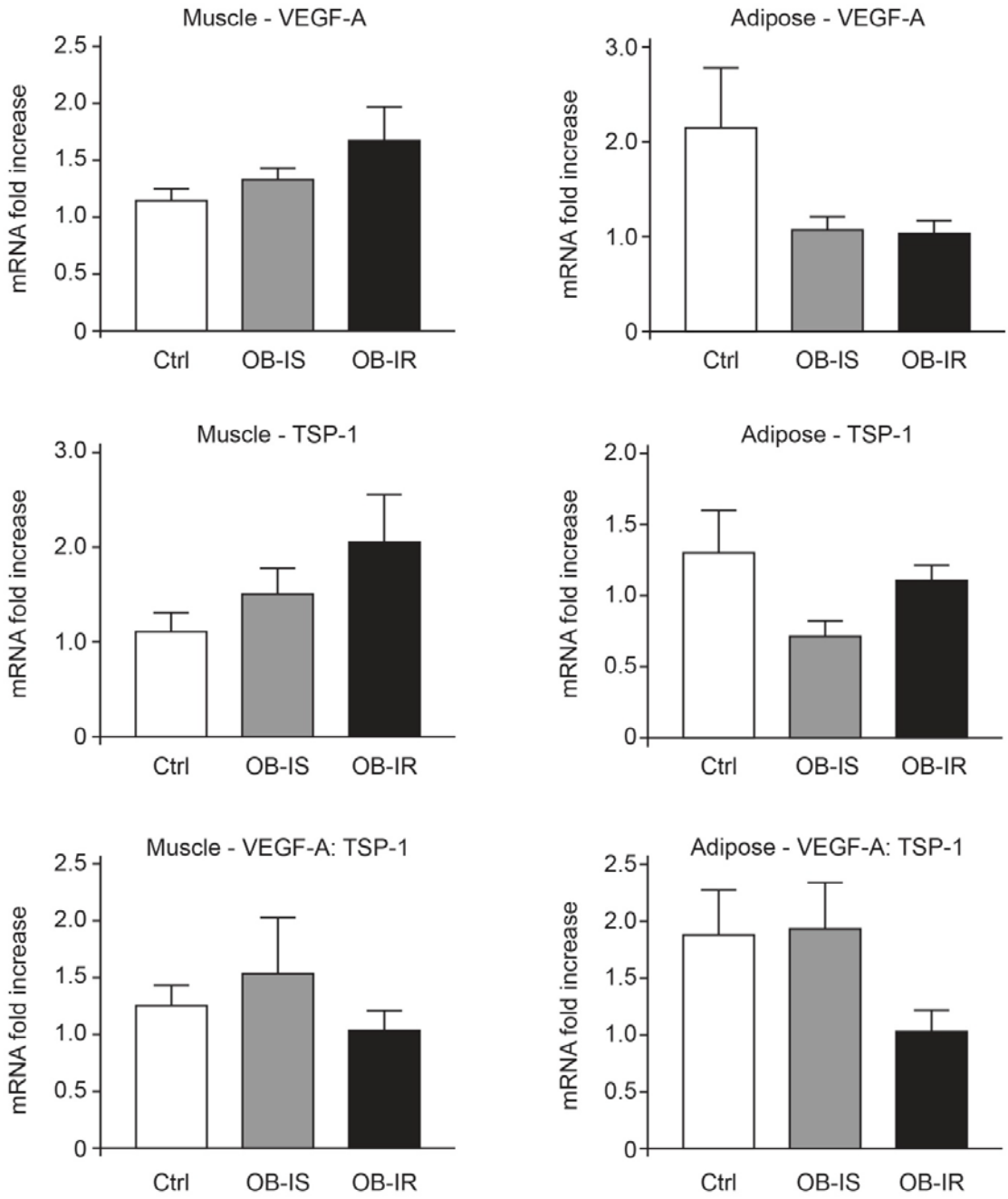

Figure A4. Effect of obesity and insulin resistance on human skeletal muscle and adipose tissue expression of VEGF-A and TSP-1. Muscle and adipose tissue were obtained from female healthy control subjects (Ctrl, n = 10, BMI<25 kg/m2, HOMA<3), obese insulin sensitive (OB-IS, n = 12, BMI>30 kg/m2, HOMA<3) and obese insulin resistant (Ob-IR, n=9, BMI>30 kg/m2, HOMA>3) female patients. VEGF-A and TSP-1 mRNA expression were measured using Taqman qPCR, and relative expression quantified relative to the housekeeping gene HPRT. Data are shown as mean ± SEM.

APPENDIX D

Angiogenesis Proteome Profiler Array – Average pixel density of all proteins.

Angio-adaptive Factor	Average Pixel Density			
	CD90+ (NG)	MyoFib (NG)	CD90+ (HG)	MyoFib (HG)
Activin A	0.0653916	0.0798531	0.0421883	0.0418316
ADAMTS-1	0.0023249	0.0055314	-0.0017389	0.0009454
Angiogenin	0.0216571	0.0461472	0.0174389	0.0366668
Angiopoietin-1	0.0087775	0.0272204	0.0125073	0.0164285
Angiopoietin-2	0.0098708	0.0150483	0.0067528	0.0121565
Angiostatin	0.0031963	0.0028313	0.0064749	0.0088242
Amphiregulin	0.0032909	0.0034265	0.0019364	0.0010769
Artemin	0.0068473	0.0069668	0.0088886	0.0041499
Coag. Factor III	0.0045861	-0.0035386	0.0018962	0.0040672
CXCL 16	0.0101491	0.0007126	0.0146247	-0.0017930
DPPIV	0.0783494	0.0225707	0.0452054	0.0078149
EGF	0.0019119	0.0027829	-0.0008411	0.0003101
EG-VEGF	0.0153446	0.0078450	0.0035638	0.0156410
Endoglin	0.0038439	0.0041321	-0.0002889	-0.0036800
Endostatin	0.0303964	0.0680864	0.0144492	0.0264744
Endothelin-1	0.0156029	0.0121290	0.0175193	0.0124572
FGF Acidic	0.0011897	0.0097481	0.0065444	0.0006766
FGF Basic	-0.0034200	-0.0008558	0.0058898	-0.0025317
FGF-4	-0.0016955	0.0028416	0.0056685	0.0043341
FGF-7	0.0023758	-0.0014838	-0.0001573	0.0123933
GDNF	0.0020356	-0.0028623	0.0024411	-0.0011333
GM-CSF	0.0072675	0.0084575	0.0078463	0.0044375
HB-EGF	0.0006003	0.0725584	0.0015689	0.0153892
HGF	0.0186810	0.0011249	0.0090952	0.0046592
IGFBP-1	0.0310422	0.0425930	0.0154933	0.0240761
IGFBP-2	0.0736251	0.2468280	0.0689911	0.3332563
IGFBP-3	0.4755432	0.9773137	0.3972009	0.9855073

IL-1β	0.0067655	0.0012681	-0.0033170	-0.0005357
IL-8	0.0524864	0.1736761	0.0686400	0.0978965
LAP (TGF-β1)	0.0121102	0.0149223	0.0113809	0.0161635
Leptin	0.0050718	0.0036870	-0.0018432	-0.0051253
MCP-1	0.5324866	0.0197446	0.6019196	0.0266398
MIP-1α	0.0093596	0.0077778	0.0062427	0.0052362
MMP-8	-0.0015772	-0.0033488	-0.0022692	0.0089031
MMP-9	0.0284462	0.0204157	0.0208253	0.0232435
NRG1-β1	-0.0021975	0.0055762	-0.0015232	-0.0054543
Pentraxin 3	0.3884454	0.1916695	0.4001466	0.1329093
PD-ECGF	-0.0092432	-0.0096929	-0.0067199	-0.0071157
PDGF-AA	0.0048299	0.0362456	0.0059483	0.0247415
PDGF-AB/PDGF-BB	0.0011916	0.0109403	0.0061476	0.0101586
Persephin	0.0103965	0.0109178	0.0070381	0.0094087
Platelet Factor 4	0.0156375	0.0155193	0.0163710	0.0158872
PlGF	0.0170328	0.0092512	0.0077567	-0.0010206
Prolactin	-0.0010915	0.0021325	0.0064767	0.0036048
Serpin B5	-0.0045352	0.0007729	0.0035638	0.0075574
Serpin E1	0.9368315	0.7497179	0.8758050	0.7069684
Serpin F1	0.2059035	0.2435516	0.2018589	0.2275393
TIMP-1	1.8585421	1.6301790	1.9083020	1.3658201
TIMP-4	-0.0007950	-0.0009645	0.0015451	0.0030335
Thrombospondin-1	0.6664168	0.6629549	0.7008770	0.5213979
Thrombospondin-2	0.0040367	0.0238508	0.0104995	0.0341220
uPA	1.4798492	2.7020231	1.6569882	2.8778939
Vasohibin	0.0110314	0.0043927	0.0070253	0.0016521
VEGF	0.1194461	0.2836843	0.1008664	0.1587447
VEGF-C	0.0053320	0.0041787	0.0024941	-0.0009097
Reference Spots	1.0029343	0.9330299	1.0511299	1.0835260
Reference Spots	1.0854860	1.0556779	1.0725495	1.0057719

Angiogenesis Proteome Profiler Array – Relative percent change in pixel density

Angio-adaptive Factor	Percent Change in Pixel Density		
	MyoFib (NG) vs. CD90+ (NG)	MyoFib (HG) vs. CD90+ (HG)	MyoFib (HG) vs. MyoFib (NG)
Activin A	122	99	52
ADAMTS-1	238	-54	17
Angiogenin	213	210	79
Angiopoietin-1	310	131	60
Angiopoietin-2	152	180	81
Angiostatin	89	136	312
Amphiregulin	104	56	31
Artemin	102	47	60
Coag. Factor III	-77	214	-115
CXCL 16	7	-12	-252
DPPIV	29	17	35
EGF	146	-37	11
EG-VEGF	51	439	199
Endoglin	107	1274	-89
Endostatin	224	183	39
Endothelin-1	78	71	103
FGF Acidic	819	10	7
FGF Basic	25	-43	296
FGF-4	-168	76	153
FGF-7	-62	-7881	-835
GDNF	-141	-46	40
GM-CSF	116	57	52
HB-EGF	12087	981	21
HGF	6	51	414
IGFBP-1	137	155	57
IGFBP-2	335	483	135
IGFBP-3	206	248	101

IL-1β	19	16	-42
IL-8	331	143	56
LAP (TGF-β1)	123	142	108
Leptin	73	278	-139
MCP-1	4	4	135
MIP-1α	83	84	67
MMP-8	212	-392	-266
MMP-9	72	112	114
NRG1-β1	-254	358	-98
Pentraxin 3	49	33	69
PD-ECGF	105	106	73
PDGF-AA	750	416	68
PDGF-AB/PDGF-BB	918	165	93
Persephin	105	134	86
Platelet Factor 4	99	97	102
PlGF	54	-13	-11
Prolactin	-195	56	169
Serpin B5	-17	212	978
Serpin E1	80	81	94
Serpin F1	118	113	93
TIMP-1	88	72	84
TIMP-4	121	196	-315
Thrombospondin-1	99	74	79
Thrombospondin-2	591	325	143
uPA	183	174	107
Vasohibin	40	24	38
VEGF	237	157	56
VEGF-C	78	-36	-22
Reference Spots	93	103	116
Reference Spots	97	94	95