

*Ambient hypoxia differentially impacts thrombospondin-1 and
thrombospondin-4 in differentiated murine myotubes*

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Abstract

Skeletal muscle microvasculature responds to stimuli with great plasticity through angioadaptation which itself is tightly regulated by a balance of pro- and antiangiogenic factors. THBS-1 has been established as the key antiangiogenic factor in skeletal muscle, and its homologue, THBS-4, has recently been identified as a proangiogenic factor in tissue other than skeletal muscle. Hypoxia has been shown to be a principal component of exercise, a potent angiogenic stimulus, yet its impact on THBS-1 and THBS-4 in myotubes has rarely been examined. My project aimed to investigate the impact of hypoxia on THBS-1 and THBS-4 in differentiated murine myotubes. The findings suggest that 1) myotubes are a source of THBS-1, 2) hypoxia greatly reduces THBS-1 and THBS-4 intracellular expression after 24 hours, and 3) hypoxia differentially impacts THBS-1 and THBS-4 in their protein stability and secretion. My research concludes that hypoxia differentially impacts THBS-1 and THBS-4 in myotubes.

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List of Abbreviations

ADAMTS – A disintegrin and metalloproteinase with Thrombospondin motifs

ANOVA – Analysis of variance

Akt – Protein Kinase B

BCA – Bicinchoninic Acid

BDNF – Brain-derived neurotropic factor

CHX – Cycloheximide

CO₂ – Carbon dioxide

COMP / THBS-5 – Cartilage oligomeric protein / Thrombospondin-5

COPD – Chronic obstructive pulmonary disorder

CT – Cycle threshold

Ctrl – Control

cDNA – complimentary deoxyribonucleic acid

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl Sulfoxide

EC – Endothelial cell

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

ER – Endoplasmic reticulum

ERK – Extracellular signal-regulated kinase

eNOS – Endothelial nitric oxide synthase

FBS – Fetal bovine serum

FGF-2 – Fibroblast growth factor-2

HIF-1 α – Hypoxia-inducible factor-1 α

HPRT – hypoxanthineguanine ribosyltransferase

HRE – Hypoxia response element

HRP – Horseradish peroxidase

HSP – heparin sulfate proteoglycan

HUVEC – Human umbilical vein endothelial cell

Hx – Hypoxia

h - hour

K – Potassium

KO - knockout

LRP1 – low density lipoprotein receptor related protein-1

MAPK – Mitogen-activated protein kinase

MEK – Mitogen-activated protein kinase kinase

MWCO – molecular weight cut-off

mRNA – messenger ribonucleic acid

mutTHBS-KI – mutant thrombospondin-4 knock-in

N₂ – Nitrogen

NO – Nitric oxide

NaCl – Sodium chloride

NaF – Sodium fluoride

Nx – Normoxia

O₂ – Oxygen

OD – Optical density

PBS – Phosphate-buffered saline

PC – Procollagen

PCR – Polymerase chain reaction

PGC-1 β – Peroxisome proliferator-activated receptor- γ coactivator-1 β

PI3K – Phosphoinositide-3-kinase

PLC γ – Phospholipase-C γ

PMSF – Phenylmethylsulfonyl fluoride

PO₂ – Oxygen partial pressure

qPCR – Real-time polymerase chain reaction

RIPA – Radioimmunoprecipitation assay

RT – Reverse transcription

SMEC – Skeletal muscle endothelial cell

sGC – Soluble guanylyl cyclase

TGF-1 β – Transforming growth factor-1 β

THBS-1 – Thrombospondin-1

THBS-2 – Thrombospondin-2

THBS-3 – Thrombospondin-3

THBS-4 – Thrombospondin-4

Type I – Properdin-like repeat

Type II – Epidermal growth factor-like repeat

Type III – Calcium-binding repeat

t_{1/2} – Half-life

VE-Cadherin – Vascular endothelial cadherin

VEGF-A – Vascular endothelial growth factor-A

VEGFR1 – Vascular endothelial growth factor receptor-1

VEGFR2 – Vascular endothelial growth factor receptor-1

WT – Wild-type

I. Introduction

I.1. The skeletal muscle, its microvasculature and the concept of angio-adaptation

Skeletal muscle is a complex organ whose physiological relevance has been well-established (Richardson et al., 1999; Wagner, 2001; Febbraio & Pedersen, 2005; Pedersen et al., 2007; Wagner et al., 2016). It is the largest organ in the human body, representing roughly 40% of our body mass, and it ensures many important physiological functions including locomotion, force production, glycaemia control, thermoregulation and myokine signaling (Fitts, McDonald & Schluter, 1991; Tickle et al., 2020; Chadderdon et al., 2012; Matsugi et al., 2016; Wagner et al., 2016; Tatsumi et al., 2002; Braun & Marks, 2015). Accordingly, many cell types can be found in the skeletal muscle tissue, namely myofibers, satellite cells, tissue macrophages, fibroblasts, pericytes, endothelial cells, neurons and lymphatic cells (Paylor et al., 2011; Rubenstein et al., 2020).

Myofibers represent the contractile units of muscle and comprise the largest cell population in this tissue (Rubenstein et al., 2020). More importantly, however, recent data suggests they also act as key regulators of skeletal muscle homeostasis as well as systemic homeostasis via myokine signaling within the muscle and other tissues such as adipose tissue, the brain and the vascular network (Boström et al., 2012; Severinsen & Pedersen, 2020; Kim et al., 2019; Delezie & Handschin, 2018; Burton & Barclay, 1986; Kivela et al., 2008; Roudier et al., 2010). For example, myofibers may act on the muscle itself by producing decorin, an autocrine-acting myokine upregulated in exercised muscle, which increases the muscle's response to exercise (Kanzleiter et al., 2014). In contrast, myofibers also produce irisin, an exercise-induced paracrine-acting myokine which causes a phenotypic change in white adipose tissue by activating the browning process (Boström et al., 2012). Moreover, myofibers have recently been shown to produce

cathepsin B, an endocrine-acting myokine which upregulates circulating brain-derived neurotrophic factor (BDNF) and is associated with improved cognitive function, in response to exercise (Moon et al., 2016). Unsurprisingly, these impressive autocrine, paracrine and endocrine signaling networks are reliant on the skeletal muscle microvasculature. The microvasculature is composed of endothelial cells (EC), which are glycolytic, selectively permeable cells that play a critical role in muscle insulin sensitivity, gas exchange and myokine signaling to and from the tissue (Wang et al., 2006; Krützfeldt et al., 1990; Quintero et al., 2006; Rubenstein et al., 2020; Adair & Montani, 2010). Functionally, the relationship between myofiber and skeletal muscle EC (SMEC) supports many skeletal muscle functions, and failure of this crosstalk between myofiber and EC may result in deleterious outcomes for the muscle (Richardson et al., 2000; Tickle et al., 2020; Tang et al., 2016; Milkiewicz et al., 2011; Roudier et al., 2013; Aiken et al., 2019).

The skeletal muscle tissue itself responds to physiological stimuli or pathological conditions with remarkable plasticity, well documented at the level of the muscle metabolic and contractile phenotypes as well as at the level of its microvasculature (Booth & Thomason., 1991; Horscroft et al., 2014; Deveci & Egginton, 2003; Mathieu-Costello, 2001; Gliemann et al., 2015). Capillaries, our smallest blood vessels, represent the site of exchange between the blood and myofibers at which the cells may exchange nutrients, gases, cellular waste and hormones. In response to physiological or pathological conditions, the capillary network can grow, stabilize, or regress (Adair & Montani, 2010; Carrow et al., 1967; Kolluru et al., 2012; Roudier et al., 2010; Aiken et al., 2019) in a process characterized as skeletal muscle angioadaptation (Zakrzewicz, Sicomb & Pries, 2002; Roudier et al., 2010; Olfert & Birot 2011). That is to say, SMEC forming the capillaries can proliferate, migrate and assemble in order to form new capillaries through angiogenesis (Ausprunk & Folkman, 1977; Gupta et al., 1999; Egginton & Birot, 2014; Hudlicka

et al., 1992; Lamallice et al., 2007), or undergo apoptosis causing the level of muscle capillarization to regress (Olfert & Birot, 2011; Olfert, 2016; Fujino et al., 2005; Malek, Olfert & Esposito, 2010). Functionally, skeletal muscle angioadaptation would ensure an optimal match between the capillary blood supply and the myofibers' metabolic needs (Marti & Risau, 1999; Olfert & Birot, 2011; Hoier et al., 2012; Tickle et al., 2020).

Angioadaptation has been well-described in the many publications put forth by the Angiogenesis Research Group and the Wagner group with respect to exercise training and detraining as well as in the context of disease states such as obesity, hypokinesia and diabetes (Birot et al., 2003; Roudier et al., 2010; Milkiewicz et al., 2011; Aiken et al., 2016; Aiken et al., 2019; Gustafsson et al., 2002; Olenich et al., 2013; Olenich et al., 2014; Malek, Olfert & Esposito, 2010). For instance, while endurance exercise training is a well-established promoter of capillary growth in skeletal muscle in healthy subjects (Olenich et al., 2013; Gustafsson et al., 2002; Birot et al., 2003; Olfert, 2006), single bouts of endurance exercise alone do not induce skeletal muscle angioadaptation (Olenich et al., 2014; Gustafsson et al., 2002). In response to short-term training (3-14 days), however, capillary growth can be detected within 14 days of moderate intensity endurance exercise training onset (Hoier et al., 2020). In contrast, the effect of detraining generates an opposite effect in the muscle microvasculature. Malek, Olfert and Esposito (2010) found capillary regression in trained rodents to occur in as early as 7 days of detraining. After 28 days of detraining, mice who exercised voluntarily for 21 days prior saw their capillary-to-fiber ratios reduced to near baseline (Olenich, 2014). Similarly, disease states such as hypokinesia, diabetes and COPD have all been shown to induce capillary regression in the skeletal muscle (Roudier et al., 2010; Aiken et al., 2019; Gouzi et al., 2012).

The maintenance of the established microvasculature is an active process requiring, at a cellular level, the survival of EC via molecular survival signals (Lee et al., 2007; Adair & Montani, 2010; Roudier et al., 2012). Angioadaptation is tightly regulated by a plethora of angioadaptive molecules in a balance of pro- and antiangiogenic factors (Hudlicka et al., 1992; Olfert & Birot, 2011; Olfert, 2016; Bornstein, 2009). Factors such as FGF-2, angiopoietin, angiomin, eNOS and the key proangiogenic factor, vascular endothelial growth factor-A (VEGF-A), can promote the expansion of the capillary network by stimulating EC proliferation, survival and migration (Carmeliet, 1999; Amaral et al., 2001; Wagner et al., 2016; Chang et al., 2003). Factors such as endostatin, vasohibin, angiotensin and the key antiangiogenic factor, thrombospondin-1 (THBS-1), can also regulate the expansion, or even promote the regression, of the capillary network by inhibiting EC migration, proliferation and survival (Bornstein, 2009; Iruela-Arispe et al., 1999; Iruela-Arispe & Dvorak, 1997; Olfert & Birot, 2011; Jiménez et al., 2000). In this manner, the balance between pro- and antiangiogenic factors can directly impact the number of capillaries being formed or pruned in the muscle.

Among all the angioadaptive molecules described in the literature known to participate in skeletal muscle angioadaptation, VEGF-A and THBS-1 are the two which have been well established as essential to this adaptive mechanism. Their critical roles have indeed been illustrated using transgenic animal models (Olfert et al., 2009; Sulaeman et al., 2020; Malek & Olfert, 2009; Delavar et al., 2014; Knapp et al., 2016; Tang et al., 2016).

1.2 The key proangiogenic factor: VEGF-A

VEGF-A is a 42KDa heterodimeric protein existing primarily in the extracellular matrix (ECM) and plasma. It was first identified by Senger et al. (1983) as vascular permeability factor, as it was first isolated from animal tumours and was found to be responsible for the increased

permeability of tumour blood vessels. It was not until 1989 that the term “VEGF” appeared in the literature and its contribution to physiological angiogenesis *in vivo* was established (Leung et al., 1989; Hanahan & Folkman, 1996; Amoroso et al., 1997; Connolly et al., 1991; Jin, Kaluza & Jakobsson, 2013). Today, the VEGF family contains 5 members, and VEGF-A, is known to have 9 isoforms through differential exon splicing: VEGF-A₁₄₅, VEGF-A₁₄₈, VEGF-A₁₆₂, VEGF-A₁₆₅, VEGF-A_{165b}, VEGF-A₁₈₃, VEGF-A₁₈₉, VEGF-A₂₀₆ and the soluble isoform VEGF-A₁₂₁ (Melincovici et al., 2018). VEGF-A₁₆₅ has been found to be expressed in endothelial cells, monocytes, vascular smooth muscle cells, cardiomyocytes, myotubes and tumour cells (Seko et al., 1999; Richardson et al., 1999; Senger et al., 1983).

VEGF-A acts on EC via two transmembrane receptors, VEGFR1 and VEGFR2 (Hojo et al., 2000; Shibuya, 2006; Gerber et al., 1998; Takahashi et al., 1999; Blanes et al., 2007; Melincovici et al., 2018). While VEGFR1 is more abundant than VEGFR2 and is involved in non-pathological VEGF-induced angiogenesis (Shibuya, 2007; Melincovici et al., 2018), the binding of VEGF-A to VEGFR2 elicits the activation of many important pathways for angiogenesis (Takahashi et al., 1999; Blanes et al., 2007; Melincovici et al., 2018). VEGFR2 is a tyrosine kinase receptor which, upon VEGF-A binding, autophosphorylates tyrosine residues on its intracellular domain leading to the activation of three pathways: the phospholipase-C γ (PLC γ) signaling pathway, the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway and the p38-Mitogen-activated protein kinase (MAPK) signaling pathway (Hoeben et al., 2004; Gerber et al., 1998; Blanes et al., 2007; Takahashi et al., 1999). The first pathway, involving PLC γ , promotes the proliferation of EC via MEK/ERK activation (Takahashi et al., 1999). The second, involving PI3K/Akt, promotes EC survival and vascular permeability through the production of eNOS (Gerber et al., 1998; Blanes et al., 2007). The third pathway, involving p38-MAPK, is responsible

for the reorganization of EC cytoskeletons, weakening cell junctions and generating a microenvironment in favour of EC migration (Koch et al., 2012; Melincovici et al., 2018).

In skeletal muscle, VEGF-A is essential for capillary maintenance and angiogenesis. Tang et al. (2004) developed *VEGFloxP^{+/+}* mice and injected the gastrocnemius of these rodents with a cre-recombinase expressing viral vector to assess changes in muscle capillarity. After 4 weeks, the VEGF-inactivated regions of the gastrocnemius exhibited a 64% decrease in capillary density and capillary-to-fiber ratio (Tang et al., 2004). Using a myofiber-specific VEGF-A deletion model, the same team also showed that 1) skeletal myocytes represent the major source of VEGF-A in the skeletal muscle tissue, 2) VEGF-A is indispensable for angiogenesis to occur in response to endurance training, and 3) VEGF-A deletion reduces the potential for exercise capacity adaptations (Tang et al., 2004; Olfert et al., 2010; Delavar et al., 2014). Skeletal muscle capillary morphology was also found to be impacted by VEGF-deficiency in this model (Baum et al., 2017). Interestingly, Lee et al. (2007) elucidated that VEGF-A not only acts through paracrine signaling but also autocrine signaling in EC as well. As mentioned, paracrine signaling by EC-neighbouring cells such as myotubes is largely responsible for promoting the differentiation, proliferation and migration of these cells to form new capillaries (Delavar et al., 2014; Sulaeman et al., 2020). Autocrine signaling however, plays an essential role for preserving the capillaries and the survival of EC (Lee et al., 2007). Lee et al. (2007) generated EC-specific VEGF KO mice by crossing VE-Cadherin-Cre transgenic mice to *VEGF^{lox/lox}* mice to generate VE-Cadherin-cre/*VEGF^{lox/+}* mice. These mice were further crossed to *VEGF^{lox/lox}* mice to generate VE-Cadherin-Cre/*VEGF^{lox/lox}* (*VEGF^{ECKO}*) mice. Adult *VEGF^{ECKO}* mice exhibited normal plasma VEGF concentrations and tissue VEGF expressions, yet these animals suffered from various systemic vascular pathologies, increased developmental lethality, impaired formation of new capillaries and, most notably,

capillary rarefaction in pre-existing networks (Lee et al., 2007). Taken together, it is then evident that VEGF-A plays a critical role in both the formation of new capillaries by angiogenesis and the maintenance of pre-existing capillary networks through two distinct signaling modalities.

I.3.1 Thrombospondins

THBS-1 belongs to a family of glycoproteins consisting of five members along with THBS-2, THBS-3, THBS-4 and THBS-5. They are secreted, calcium-binding proteins which localize to the ECM. Unlike other matricellular proteins however, they do not all play structural roles in the microenvironment (Sage & Bornstein, 1991). Instead, these proteins participate in various cell-cell and cell-ECM interactions, interacting with various cell-surface receptors and a variety of ECM proteins such as collagens, proteases and growth factors (Sage & Bornstein, 1991). Despite a high degree of homology between the members of this family, thrombospondins can be divided into two subgroups based on their domain structure. Subgroup A comprises the homotrimeric members, THBS-1 and THBS-2, while subgroup B comprises the homo- or heteropentameric members, THBS-3, THBS-4 and THBS-5 (Bornstein, 2009; Adams & Lawler, 2011).

I.3.2 THBS-1 and THBS-2

THBS-1 and THBS-2 are ~150KDa homotrimeric glycoproteins composed of 1,170 and 1,172 amino acids, respectively, and are among the first antiangiogenic proteins to be identified (Lawler et al. 1978, Good et al., 1990; Bornstein et al., 1991; Volpert et al., 1995). THBS-1 was first isolated and characterized from thrombin-treated platelets by Lawler and his team (1978). The first cell types found to secrete THBS-1 were cultured primary bovine aortic EC (McPherson et al., 1981) and cultured human umbilical vein EC (HUVEC) (Mosher et al., 1982). Soon after its

discovery, a second expressed gene was discovered by the sequencing of a mouse genomic clone. This gene was clearly homologous to THBS-1, but the expressed cDNA was then sequenced and revealed to be a different gene, that of THBS-2 (Bornstein 1991). Today, cell types known to produce and secrete THBS-1 and THBS-2 include, but are not restricted to, monocytes, CD34⁺ EC, fibroblasts, cardiomyocytes, smooth muscle cells and myotubes (Lawler et al., 1987; Mosher et al., 1982; Panetti et al., 1997; Bornstein et al., 1991; Iruela-Arispe et al., 1993; Isenberg et al., 2005; Bornstein, 2009; Yadav et al., 2014; Xing et al., 2017).

I.3.3 THBS-1 and THBS-2 structure and antiangiogenic function

The antiangiogenic potential of THBS-1 was first detected by Good et al. (1990) who isolated THBS-1 from baby hamster kidney cells and found it inhibited the migration of bovine adrenal capillary EC *in vivo* as well as EC proliferation and migration *in vitro*. Around the same period, Taraboletti et al. (1990) discovered that THBS-1 also exerts an angiogenic effect on bovine aortic EC and pulmonary capillary EC. For THBS-2, the first evidence of antiangiogenic activity came from Volpert and his team in 1995 (Volpert et al., 1995). Using recombinant murine THBS-2 as well as bovine THBS-2, the team elucidated its ability to block the migration of EC *in vitro* and rat corneas *in vivo*.

Today, both THBS-1 and THBS-2 are known to bear a multitude of antiangiogenic properties made possible by their complex structure. THBS-1 and THBS-2 have a heparin-binding domain at the N-terminal, followed by an oligomerization domain including interchain disulfide bonds (coiled-coil domain), a pro-collagen (PC) homology domain, three properdin-like (type I) repeats, three EGF-like (type II) repeats, and seven calcium-binding (type III) repeats, capable of binding a total of 26 Ca²⁺ ions, near its lectin-like globular C-terminal domain which functions as a cell-binding domain (Iruela-Arispe et al., 1999; Brown & Frazier, 2001; Bornstein, 2009; Zhao

et al., 2017). Due to these various functional domains, THBS-1 and THBS-2 can regulate angiogenesis through a number of interactions in the ECM, though it is important to note that not all these interactions are shared between the two proteins.

Firstly, the heparin-binding domains of THBS-1 & THBS-2 bind heparin-sulfate proteoglycans, which act as coreceptors for the low-density lipoprotein receptor-related protein-1 (LRP1). LRP1 acts as a scavenger receptor which clears the ECM of complexes of THBS-1 or THBS-2 with matrix metalloproteinase (MMP)-2, MMP-9 and VEGF-A (Godyna et al., 1995; Mikhailenko et al., 1995). This causes a reduction in VEGF-A abundance and protease activity leading to an inhibition of angiogenesis. More so, the N-terminal of THBS-1 and THBS-2 bind a collection of integrins found on EC membranes. For example, both THBS-1 and THBS-2 bind $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins, receptors important for microvascular EC cell-cell adhesion and spreading, on their N-terminal which inhibits EC migration and chemotaxis (Chandrasekaran et al., 2000). In contrast, only THBS-1 has been shown to bind $\alpha 4\beta 1$, an integrin which supports venous EC adhesion and mediates microvascular EC chemotaxis (Calzada et al., 2004).

The type I repeats found on THBS-1 and THBS-2 also serve important antiangiogenic functions. Both proteins are capable of binding CD36 on EC surface membranes and induce apoptosis of EC through a signaling pathway mediated by p59fyn and p38-MAPK activation (Jimenez et al., 2000). Cleavage of these type I repeats by an ADAMTS proteinase produces a 36kDa fragment which initiates the pro-apoptotic signaling cascade of caspases (Jimenez et al., 2000). THBS-1 has also been shown to displace matrix-bound FGF-2 by interaction with FGF-2 on its type I repeats and PC domain, inhibiting the proliferation of EC (Margosio et al., 2003). This ability is not shared with THBS-2 as the activating sequence on the type I repeat differs slightly between the two proteins (Ribeiro et al., 1999). This difference also accounts for the exclusive

ability of THBS-1 to activate latent TGF-1 β through complex forming with latency-associated peptide (Ribeiro et al., 1999). The type III repeats of THBS-1 and THBS-2 have been suggested to support FGF2 binding, but these findings are recent and have not been as well-established as those mentioned above (Rusnati et al., 2019).

The last notable antiangiogenic function of THBS-1 occurs via its C-terminal. Through this domain, THBS-1 is capable of binding CD47, which recognizes VVM a sequence on the C-terminal and has been established as an antagonist receptor of NO signaling (Brown & Frazier, 2000; Isenberg et al., 2006). NO production by eNOS is a downstream response of VEGF-A binding to VEGFR2 on EC surface membranes and is an important factor for vasodilation, EC proliferation and migration. Binding of THBS-1 to CD47 is capable of completely blocking NO/cGMP accumulation in EC by inhibiting soluble guanylate cyclase (sGC) and thus strongly inhibiting the VEGF-A-mediated angiogenic responses (Isenberg et al. 2005b; Isenberg et al., 2008). As THBS-2 lacks the specific VVM sequence on its C-terminal required for CD47 binding, it does not share this function with THBS-1.

1.3.4 Pentameric thrombospondins

THBS-3, THBS-4 and THBS-5 are the pentameric members of the thrombospondin family (Bornstein, 2009; Adams & Lawler, 2011). They are ~105KDa secreted glycoproteins, though some recent reports suggest intracellular functions at the level of the endoplasmic reticulum and golgi apparatus for certain members (Lynch et al., 2012; Brody et al., 2016). Unlike THBS-1 and -2, however, there is no evidence to support antiangiogenic function for these members of the thrombospondin family. (Hecht et al., 1998; Bornstein, 2009; Brody et al., 2016, Stenina-Adognravi, 2014). This is likely due to the lack of the PC domain and type I repeats in their structure. Despite this, the pentameric thrombospondins still participate in many important ECM-

cell functions such as muscle membrane stability, tendon organization and wound healing (Adams & Lawler, 2011; Schips et al., 2019; Posey et al., 2008; Vanhoutte et al., 2016; Frolova et al., 2014 Kassem et al., 2019).

I.3.5 THBS-3

The THBS-3 gene was first identified by Vos et al. (1992) shortly after the discovery of THBS-2 through sequencing both mouse and human genomes. The team described this new gene to be highly homologous to that of THBS-1 and THBS-2 (58% and 59%, respectively) and concluded that this gene codes for THBS-3 protein. Though it was the first of the pentameric thrombospondins to be discovered, little research has been conducted on this protein since its characterization, with less than 50 hits on Pubmed regarding THBS-3 (<https://pubmed.ncbi.nlm.nih.gov/?term=thrombospondin-3>). What is known about THBS-3 today is that it exists primarily in developing bone, skin and cartilage (Iruela-Arispe et al., 1993; Hankenson et al., 2005; Posey et al., 2019), and has been suggested to participate in the development of bone and cartilage (Iruela-Arispe et al., 1993; Posey et al., 2019) as well as aggravate cardiac pathologies by destabilizing various integrins on the sarcolemma of cardiomyocytes (Schips et al., 2019).

I.3.6 THBS-4

Possibly the most notable of the pentameric thrombospondins, THBS-4 was first identified by Lawler's team in 1993 (Lawler et al., 1993). In the years following its discovery, THBS-4 was found to be expressed in tendon, developing bone, retinal layers, the nervous system, the microvasculature and skeletal muscle (Hauser et al., 1995; Arber & Caroni, 1995; Carron et al., 2000; Si et al., 2003; Stenina-Adognravi et al., 2003; Vanhoutte et al., 2016; Stenina-Adognravi

& Plow, 2019). Like the other thrombospondins, THBS-4 is largely involved in ECM organization and cell-ECM interactions in tissues such as tendon, cartilage, cardiac and skeletal muscle, though some reports suggest it serves intracellular functions as well (Brody et al., 2016; Lynch et al., 2012). Indeed, mounting evidence suggests THBS-4 functions in the cell as an endoplasmic reticulum (ER)-stress response protein as well as a chaperone for the secretion of other proteins to the ECM (Adams & Lawler, 2011; Lynch et al., 2012; Vanhoutte et al., 2016; Brody et al., 2016). THBS-4 also participates in tissue remodelling events such as wound healing, myotendinous junction formation and more recently, has been suggested to promote angiogenesis (Frolova et al., 2014; Muppala et al., 2015; Muppala et al., 2017; Stenina-Adognravi & Plow, 2019).

1.3.7 Potential proangiogenic functions of THBS-4

The first evidence of THBS-4 involvement in vascular remodelling came from Frolova et al. (2014) with the use of THBS-4-null mice. The myocardia of these mice were subjected to pressure overload by transverse aortic constriction. Indeed, THBS-4-null mice exhibited a dramatic decrease in microvessels around the myocardium when compared to wild-type (WT) controls (Frolova et al., 2014). The angiogenic potential of THBS-4 was then tested both *in vitro* and *in vivo* (Muppala et al., 2015). The team first injected matrigel plugs containing 10ng/mL FGF in WT C57Bl/6 mice and THBS-4-null mice. After 7 days, immunohistochemical staining of the plugs for CD31⁺ EC revealed that the plugs excised from THBS-4-null mice had dramatically lower EC counts compared to WT controls. They also found tumour angiogenesis to be inhibited in THBS-4-null mice (Muppala et al., 2015). The researchers then isolated lung EC from WT, THBS-4-null, and mutant-THBS-4 knock-in (mutTHBS-4-KI) mice and assessed EC migration, proliferation and adhesion. Briefly, mutTHBS-4-KI mice express a P387 THBS-4 variant which has been shown to induce more extensive THBS-4 signaling than the wildtype A387 variant

(Pluskota et al., 2005; Stenina-Adognravi et al., 2005). In their *in vitro* assessments, the team showed that 1) WT EC migration is increased in THBS-4-coated Boyden chamber wells versus uncoated wells, 2) THBS-4-null EC exhibit a nearly 50% decrease in migrative potential, and 3) THBS-4-null EC proliferated 40% slower than WT EC 48 hours (h) after plating. The same team later evidenced the role of THBS-4 in mediating TGF- β -induced angiogenesis (Muppala et al., 2017).

1.3.8 THBS-5

THBS-5, also known as cartilage oligomeric matrix protein (COMP), was the last protein to be added to the thrombospondin family some time after it was cloned and sequenced by Hedbom et al. (1992). THBS-5 was originally thought to exist only in cartilage tissue but its prevalence in tendon, ligament and blood vessels was then elucidated, supporting the change in nomenclature from COMP to THBS-5 (Hecht et al., 1998; Sodersten et al., 2006). THBS-5 has been extensively studied in the context various pathologies such as sclerosis, osteoarthritis, rheumatoid arthritis, liver fibrosis, pulmonary fibrosis and some cancers, to name a few (Hecht et al., 1998; Jordan, 2005; Magdaleno et al., 2016; Englund et al., 2016; Papadakos et al., 2019). This said however, all studies having investigated THBS-5 as an angioadaptive protein have not characterized a role for this protein in the adaptive mechanism.

Rationale for focusing on THBS-1

While both THBS-1 and THBS-2 serve a multitude of antiangiogenic functions, a case can be made for THBS-1 as the key antiangiogenic factor in skeletal muscle through the use transgenic animal models. Malek & Olfert (2009) have shown that THBS-1-null mice have 1) greater muscle capillarization, 2) improved muscle force production, and 3) increased exercise running capacity

compared to wild-type controls (Malek & Olfert, 2009). This important study illustrates the key role played by THBS-1 in the skeletal muscle tissue and suggest that no other anti-angiogenic molecules were able to compensate for the deletion of THBS-1, including THBS-2. In line with Malek & Olfert (2009), Dr. Isenberg's lab has also illustrated the regulation of angioadaptation by THBS-1 *in vitro* using a THBS-1-null muscle explant model (Ridnour et al., 2005). Briefly, they embedded pectoralis muscle explants from wild-type and THBS-1-null mice into a collagen gel and followed the migration of vascular cells; as expected, vascular explant outgrowth was higher in THBS-1-null conditions (Ridnour et al., 2005). More so, the administration of a THBS-1 mimetic (ABT-150) with a mini-osmotic pump in mice has been shown to result in reduced skeletal muscle capillarity after 14-day administration compared to vehicle-only treated animals (Audet et al., 2013). In contrast, though THBS-2-null mice have also been utilized in previous studies (Kyriakides et al., 1998), this model has not been assessed at the level of the skeletal muscle and thus no supporting data exists for its essentiality in skeletal muscle angioadaptation.

Rationale for focusing on THBS-4

Of the pentameric thrombospondins, THBS-4 is evidently the most relevant to this study and, like THBS-1, its relationship with skeletal muscle and angioadaptation has been best described through the use of transgenic animals. Notably, THBS-4-null mice 1) experience skeletal muscle membrane destabilization and muscular dystrophy 2) exhibit a drastic decrease in the ECM of oxidative muscles resulting in significantly lower muscle mass, and 3) EC exhibit reduced proliferative capacity and migration both *in vivo* and *in vitro* (Vanhoutte et al., 2016; Muppala et al., 2015; Stenina-Adognravi, & Plow, 2019). Not to mention, THBS-4 is the only pentameric thrombospondin expressed in skeletal muscle capillaries (Vanhoutte et al., 2016), making it of particular interest to the study of skeletal muscle angioadaptation.

1.3.9 THBS-1 and THBS-4 expression in skeletal muscle.

As previously mentioned, myofibers represent the largest cell population in the skeletal muscle, and studies have shown that myofibers represent a major source of VEGF-A in the muscle both at rest and in response to exercise (Biro et al., 2003; Breen et al., 2008; Olfert et al., 2009; Rich et al., 2017). Far fewer studies have investigated the expression of THBS-1 and THBS-4 in the skeletal muscle (Olfert et al., 2006; Kivela et al., 2008; Malek & Olfert, 2009; Frazier et al., 2011; Roudier et al., 2010; Roudier et al., 2013; Audet et al., 2011; Olenich et al., 2013; Slopach et al., 2014; Hoier et al., 2012; Hoier et al., 2020; Suarez-Calvet et al., 2020; Frolova et al., 2014; Vanhoutte et al., 2016) and even less in skeletal muscle cells (Iruela-Arispe et al., 1993; Adams & Lawler, 1994; Adams et al., 1999; Anilkumar et al., 2002; Yadav et al., 2014; Vanhoutte et al., 2016).

At the whole muscle level, THBS-1 response has been characterized in the contexts of single-bout exercise, short term training, chronic exercise, detraining, hindlimb unloading, disease states as well as in response to hypoxia (Olenich et al., 2013; Olfert et al., 2006; Slopach et al., 2014; Hoier et al., 2012; Audet et al., 2011; Olenich et al., 2014; Frolova et al., 2014; Roudier et al., 2010; Roudier et al., 2013). Firstly, findings from Olenich et al. (2013) suggest that a single 1 h bout of exercise induces a drastic increase in THBS-1 protein expression 12 h post-exercise. At the mRNA level, the THBS-1 response to exercise is seen immediately post-exercise exhibiting a 3.5-fold increase at the end of the 1 h treadmill running protocol (Olfert et al., 2006). Indeed, the induction of THBS-1 mRNA and protein in a single bout of exercise reflects the previously mentioned outcome in which a single bout of exercise does not induce capillary growth, despite its strong stimulatory effect on many angiadaptive factors (Olenich et al., 2013).

In response to short-term training, THBS-1 protein expression in the muscle is increased after 2 hours after a single 1-hour exercise bout and remains elevated at 7 days (Slopach et al., 2014). However, this response is nearly abolished after 10 days of exercise training as THBS-1 protein expression returns to basal levels (Slopach et al., 2014). Accordingly, this loss of THBS-1 response in the muscle allows the angioadaptive balance to tip towards proangiogenic signaling and is permissive to angiogenesis. Interestingly, however, after 4 weeks of chronic exercise training, the THBS-1 mRNA response to a single bout of exercise seems to rise again (Hoier et al., 2012). At 8 weeks of chronic exercise, the THBS-1 mRNA response to a single bout of exercise is then fully restored, suggesting that THBS-1 may regulate the onset of skeletal muscle angiogenesis in response to exercise both in trained and untrained muscle (Olfert, et al., 2006). Audet et al., (2011) also examined skeletal muscle THBS-1 as well as VEGF-A protein expression in selectively bred high-aerobic capacity mice. Namely, this group focused on the soleus, gastrocnemius and plantaris muscles of these mice, the first being more oxidative and the latter two more glycolytic (Audet et al., 2011). Their observations interestingly depicted an association between muscle metabolic profile and angioadaptive protein expression both at baseline and after acute exercise. The more oxidative muscle, the soleus, exhibited no differences in VEGF-A and THBS-1 protein expression between selectively bred high aerobic capacity mice and controls at baseline nor after acute exercise, though in the more glycolytic muscle, the plantaris, these mice had 79% higher VEGF-A and 39% lower THBS-1 expression at baseline and saw a 28% increase in VEGF-A and a 50% increase in THBS-1 expression following acute exercise in comparison to controls (Audet et al., 2011).

In the context of detraining, THBS-1 protein has been found to be expressed at different timepoints in separate muscles (Olenich et al., 2014). In the plantaris of mice, THBS-1 protein expression is increased 3-fold after a single day of detraining and remains elevated at 7 days. In the soleus and gastrocnemius muscles, THBS-1 protein expression was only found to be elevated after 7 days of detraining, to a lesser extent than in the plantaris muscle (Olenich et al., 2014), again suggesting that a relationship exists between the muscle's metabolic profile and its angioadaptive response to exercise training (Audet et al., 2011; Olenich et al., 2013; Frolova et al., 2014). Hindlimb unloading experiments highlight similar findings as well. THBS-1 protein expression in rodent soleus muscle is elevated after 5 days and peaks at 7 days of unloading, whereas in the plantaris, THBS-1 expression remains unchanged after 7 days of unloading versus controls (Roudier et al., 2010).

THBS-1 has also been investigated in the context of hypoxia and ischemia (Olfert et al., 2006; Roudier et al., 2013). Olfert et al. (2006) investigated THBS-1 expression in response to acute or chronic exercise in both room air and chronic ambient hypoxia. Briefly, the team showed that muscle THBS-1 mRNA expression 1) is increased 3 to 4-fold from an acute bout of exercise, 2) sees its acute exercise-induced increase diminished with short-term exercise training, and 3) as mentioned previously, sees its acute exercise-induced increase restored after 8 weeks of chronic training (Olfert et al., 2006). In chronically hypoxic sedentary mice, however, Olfert et al. (2006) observed strong reductions in both basal and exercise-induced expressions of THBS-1 mRNA in the gastrocnemius muscles of these mice compared to room air controls (Olfert et al., 2006). Another study by Roudier et al., (2013) observed increased THBS-1 expression in the

gastrocnemius of mice following ischemic injury as well as in the ischemic muscle of patients with peripheral artery disease.

Though no studies have yet investigated the angioadaptive role of THBS-4 in skeletal muscle, its expression in muscle seems to share a relationship with muscle metabolic profile (Frolova et al., 2014; Vanhoutte et al., 2016). THBS-4 protein has been shown to be more abundant in the soleus than the gastrocnemius, having a 2-fold greater expression (Frolova et al., 2014). Furthermore, Frolova et al. (2014) found that oxidative muscles such as the soleus exhibit greater tendon, sarcolemmal and ECM abnormalities in THBS-4-null mice compared to less oxidative muscles like the gastrocnemius. Expression of THBS-4 in skeletal muscle has also been reported to protect the tissue against muscular dystrophy and contribute to grip strength in mice (Vanhoutte et al., 2016; Frolova et al., 2014). However, no studies have yet elaborated on the involvement of THBS-4 in skeletal muscle angioadaptation in response to exercise or physiological stress.

The major component of muscle are the myofibers. Yet, how THBS-1 and THBS-4 are regulated, expressed or secreted remains largely unknown. The majority of published data on THBS-1 in skeletal muscle cells stems from J.C Adams' lab and focuses primarily on its involvement in cell spreading and fascin spike organization (Adams & Lawler, 1994; Adams et al., 1999; Anilkumar et al., 2002). Yadav et al. (2014), however, conducted a thorough study of hypoxic myotube THBS-1 mRNA expression in response to PGC-1 β , a suggested novel transcription cofactor in the regulation of THBS-1 in skeletal muscle. They found that C2C12 myotube THBS-1 transcript is downregulated in hypoxia and is associated with a downregulation of PGC-1 β expression (Yadav et al., 2014). Though this is the first evidence of THBS-1 regulation in hypoxic skeletal muscle cells, the data only suggests a relationship between PGC-1 β and THBS-1 transcript, where more factors are certainly involved at the post-translational, protein and

secretory level. Indeed, a pubmed search consisting of the word “thrombospondin” and either “myotube”, “myofiber”, “C2C12” or “skeletal muscle cell” does not yield any further data on the expression and regulation of THBS-1 in skeletal muscle cells. More research is thus required to elucidate the regulation of THBS-1 mRNA and protein in the myofiber.

Likewise, the expression, regulation and secretion of THBS-4 in the myofiber is largely unknown. Only a few studies in the current literature suggests myofibers produce and secrete THBS-4 to the ECM (Lynch et al., 2012; Brody et al., 2016; Vanhoutte et al., 2016). One study suggests that both THBS-1 and THBS-4 can regulate the endoplasmic reticulum (ER) stress response in skeletal muscle cells (Lynch et al., 2012) due to their close relationship with Ca^{2+} ions both intracellularly and in the ECM (Lynch et al., 2012; Brody et al., 2016). Indeed, this relationship has been elucidated in renal cell carcinomas, in which low Ca^{2+} concentrations were shown to retain THBS-1 in the ER, and restoration of adequate Ca^{2+} levels permitted the secretion of THBS-1 into the ECM (Veliceasa et al., 2007). How exercise and physiological stimuli may impact the expression of THBS-4 in myofibers however, is yet to be investigated.

1.4 Why study muscle hypoxia?

Oxygen diffusion to the skeletal muscle plays a critical role to the function and health of the tissue (Arthur et al., 1992; Powers et al., 1993; Chang et al., 2003; Breen et al., 2008). At the systemic level, hypoxia can be observed in high altitude environments in which ambient hypoxia is present (Horscroft et al., 2014). Ambient hypoxia is characterized as a lack of oxygen diffusion to the system due to ambient air of low O_2 partial pressure. Interestingly, ambient hypoxia seems to exert a rather minor effect on the intramuscular PO_2 as elucidated by Richardson and his team (2006). The team assessed myoglobin desaturation as a marker of O_2 availability within the muscle using proton nuclear magnetic resonance spectroscopy at a high field strength with 30-minute

extended signal averaging (Richardson et al., 2006). Their results found that intramuscular PO₂ in ambient normoxic air in humans sits at 34 ± 6 mmHg as opposed to 23 ± 6 mmHg under hypoxic conditions (10% inspired O₂ for 30 minutes). In contrast, exercise seems to exert a more severe hypoxic effect in contracting muscles (Richardson et al., 1995; Richardson et al., 2000). Findings from these studies reflect a physiological hypoxia at the level of the muscle. Physiological, or exercise-induced hypoxia is characterized as low oxygen diffusion at the level of the tissue as a consequence of physiological stress (Dempsey & Wagner, 1999; Richardson et al., 2000). Altogether, this suggests that muscle may be resilient to changes in ambient oxygen availability, though as Olfert et al. (2006) observed, exercise poses a stronger hypoxic stimulus to the muscle which may be further exacerbated by ambient hypoxia. Though very difficult to evaluate the individual impact of each stressor in exercise on the muscle tissue, *in vitro* models of muscle cells have been shown to be a promising approach for investigating the effect of hypoxia specifically on the myofiber (Yadav et al., 2014; Aiken et al., 2016; Fox, Walsh & Mulhall, 2018).

A common approach to studying myofibers *in vitro* is through the use of C2C12 myotubes (Nedachi, Fujita & Kanzaki, 2008; Vanhoutte et al., 2016; Matsugi et al., 2016; Yadav et al., 2014; Bensaid et al., 2019). C2C12 cells are immortalized mouse myoblast subclones stemming from the C2 myoblast line first isolated by Yaffe and Saxel in 1977 from the legs of 3H mice (Yaffe & Saxel, 1977). Today, these cells are widely available and have become well-established as a model for studying muscle physiology. As myoblasts, they have been used as a model for studying myoblasts and satellite cells *in vitro* such as in the contexts of proliferation, differentiation and regeneration (Osana et al., 2020; Cui et al., 2018; Clazia et al., 2020). Furthermore, using specific but simple media supplementation techniques, C2C12 myoblasts can be differentiated into

contractile myotubes. These C2C12 myotubes have become a standard model for studying myofibers *in vitro* due to their ability to contract in culture and their responses to physiological and pathological stressors, which have been comparable to *in vivo* findings in many cases (Nedachi, Fujita & Kanzaki, 2008; Yadav et al., 2014; Vanhoutte et al., 2016). More so, evidence suggests they are sensitive to hypoxia, making them a viable and easily reproducible model for studying the effect of hypoxia on myotube angiadaptive signaling *in vitro* (Yadav et al., 2014; Bensaid et al., 2019).

The current state of knowledge on skeletal muscle suggests THBS-1 expression may be regulated by hypoxia at the level of the myofibers' intracellular expression (Yadav et al., 2014; Olfert et al., 2006). However, being a secreted protein, it is important to note that no investigation has yet revealed the effect of hypoxia on its protein expression in, and secretion by, myotubes. In the case of THBS-4, no study has yet investigated the effect of hypoxia on myofiber expression both intracellularly and as a secreted protein. Though, given the recent evidence of its proangiogenic potential (Frolova et al., 2014; Muppala et al., 2015; Stenina-Adognravi et al., 2019), its apparent relationship with oxidative tissue (Frolova et al., 2014; Vanhoutte et al., 2016) and its relatively exclusive expression in skeletal muscle capillaries compared to THBS-3 and THBS-5 (Frolova et al., 2014), its role in skeletal muscle angiadaptation seems worthy of investigation.

I.5 Conclusion

Skeletal muscle exhibits great plasticity in response to physiological and pathological conditions at the level of its contractile phenotype, metabolic profile and its microvasculature. Skeletal muscle angiadaptation, the process in which the muscle capillaries form or regress, is a

complex process that is tightly regulated by a balance of pro- and antiangiogenic factors. The expression and regulation of some factors have been linked to the oxygen availability within the muscle, such as in the cases of moderate-to-high intensity aerobic exercise and ischemic diseases (Olfert et al., 2006; Hoier et al., 2020; Milkiewicz et al., 2011; Roudier et al., 2013). However, much of this research has focused on the proangiogenic side of the balance. While THBS-1 has been well established as the key antiangiogenic factor in skeletal muscle angioadaptation through the use of transgenic animal models (Malek & Olfert, 2009; Ridnour et al., 2005), only one study has investigated its expression and regulation in the myofiber in response to hypoxia (Yadav et al., 2014). As mentioned above, myofibers represent the largest cell population in the skeletal muscle tissue and are known to contribute to the skeletal muscle angioadaptive balance via the expression and secretion of factors such as VEGF (Delavar et al., 2014). Thus, characterizing the expression, regulation and secretion of THBS-1 by the myofiber, specifically, is of critical importance to the study of skeletal muscle angioadaptation and muscle physiology. Additionally, recent findings from Frolova et al. (2014) and Muppala et al. (2015, 2017) suggest that THBS-4 may be another thrombospondin of interest in the study of angioadaptation, thus placing a greater emphasis on the investigation of thrombospondins in the myotube. C2C12 myotubes are a well-established model for the study of myotubes *in vitro* and have been shown to respond to hypoxia with great resemblance to skeletal muscle cells *in vivo* (Yadav et al., 2014; Vanhoutte et al., 2016). Thus, the purpose of my MSc project is to investigate the impact of hypoxia on the expression and secretion of THBS-1 and THBS-4 in differentiated C2C12 myotubes and contribute to our understanding of myotube-specific thrombospondins in the context of hypoxia.

II. STUDY AIMS

II.1 Aim #1

To characterize the impact of ambient hypoxia on THBS-1 and THBS-4 expression and secretion in differentiated murine myotubes. Additionally, to confirm the reductive effect of hypoxia on THBS-1 mRNA in these cells.

Hypothesis: Hypoxia will repress THBS-1, but not THBS-4 protein expression and secretion, in differentiated murine myotubes, and THBS-1 mRNA will also be repressed in hypoxic conditions.

Rationale:

Olfert et al. (2006) found THBS-1 mRNA to be decreased in whole rodent muscles subjected to chronic hypoxia *in vivo*. Yadav et al. (2014) elucidated the impact of a hypoxia-mimetic drug on PGC-1 β expression and found a link between PGC-1 β expression and THBS-1 transcription. Neither of these studies examined the direct impact of ambient hypoxia on THBS-1 mRNA nor protein expression in the myofibers, however. This said, it is very likely that ambient hypoxia will result in a loss of THBS-1 expression both at the level of its mRNA and intracellular and secreted protein.

II.2 Aim #2

To investigate the effect of hypoxia on the protein turnover of THBS-1 and THBS-4 protein and determine whether they are differentially impacted by hypoxia. Additionally, to establish the half-life of these proteins in C2C12 myotubes.

Hypothesis: Hypoxia will impact the stability of THBS-1 but not THBS-4 protein after 90-minute exposure.

Rationale:

Protein turnover is critical to cell survival, and in the case of hypoxia, it must favor proangiogenic proteins in order to shift the angioadaptive balance and ensure adequate adaptation can occur to ultimately increase oxygen diffusion to the myofibers. Circumstantial evidence suggests hypoxia may stabilize certain proteins involved in angiogenesis (Man et al., 2018; Chen et al., 2020), thus we sought to investigate whether hypoxia may also impact the protein stability of THBS-1 and THBS-4 in differentiated C2C12 myotubes.

III. Methods

III.1 Cell culture model. Immortalized mouse C2C12 myoblasts (CRL_1772, Lot:70024392, P5-P8; ATCC, Manassas, VA, USA) will be cultured in 5% CO₂, 21% O₂ at 37°C; cultured in growth media (DMEM with 10% FBS, Cat. #080-150; Wisent, Montreal, QC, Canada + 1% Penicillin/Streptomycin, Cat. #450-200-EL; Wisent, Montreal, QC, Canada) until they reach 95% confluence (roughly 6 million per T75 flask). Cells will then be plated onto 6-well plates at a seeding density of 258,333 cells per well. Once cells reach 95% confluence (775,000 cells per well), differentiation will be induced by replacing the growth media with differentiation media (DMEM with 2% Horse Serum, Cat. #065-210; Wisent, Montreal, QC, Canada + 1% Penicillin/Streptomycin, Cat. #450-200-EL; Wisent, Montreal, QC, Canada) and cells will be allowed to differentiate for 6-7 days. Fully differentiated myotubes will then be subjected to hypoxia and or pharmacological inhibitors for up to 24 hours.

III.2 Normobaric hypoxia exposure. Normobaric hypoxia incubation is achieved in a Forma Series II Water Jacketed CO₂ Incubator (Cat. #3130; ThermoFisher, Burlington, ON, Canada). Cells are incubated in a gas mixture of 5% CO₂, 1% O₂ and 94% N₂ for up to 24 hours at 37°C, resulting in an estimated pericellular O₂ concentration of roughly 50µmol/L per well (Wenger et al., 2015).

III.3 Cellular protein isolation and quantification. Following hypoxic incubation, C2C12 protein extracts will be acquired by washing cells with ice-cold PBS (Cat. #311-013-CL, Wisent, Montreal, QC, Canada) three times then lysed with lysis buffer composed of 50mM Tris base, 100mM NaCl, 1% Triton-X, 1% sodium deoxycholate, 5mM EDTA, 1 tablet phosphatase inhibitor (Roche, Mississauga, Canada, Cat. #04 906 845 001), 1 tablet protease inhibitor (Roche, Mississauga, Canada, Cat. #11 836 153 001), 50µL NA₃VO₄, 50µL NaF and 50µL PMSF. Cells

are then scraped, and protein lysates are collected. Lysates are then centrifuged at 15,000 x *g* for 15 minutes and supernatants are collected for protein concentration measurement. 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.

III.4 Cell media concentration. Following hypoxic incubation, media was collected in tubes and protein was extracted using 50K MWCO Pierce Protein Concentrators (Cat. #88539, Thermo Scientific, Oakville, ON, Canada). Media samples were spun in a swinging-bucket centrifuge at 5000 x *g* for 20 minutes then collected and stored at -80°C for later use.

III.5 Western blot analysis. Immunoblot analysis was conducted on protein extracts from C2C12 myotubes. Intracellular protein samples were loaded at a concentration of 10µg/µL and concentrated media samples were loaded at a concentration of 100µg/µL with 6µL denaturing buffer (2.69mM sucrose, 0.28M sodium dodecyl sulfate, 2.84M β-mercaptoethanol, 14µM bromophenol blue, 0.1M Tris pH 6.8, H₂O) and supplemented with RIPA buffer without inhibitors to reach a final loading volume of 30uL. Blots were probed with the following primary antibodies: αβ-tubulin (Cat. #2148; Cell Signaling Technology, Beverly, MA, USA), β-actin (Cat #4967, Cell Signaling Technology, Beverly, MA, USA), THBS-1 (Cat #MA5-13398, Invitrogen, Oakville, ON, Canada,) and THBS-4 (Cat #ab263898, Abcam, Boston, MA, USA). After incubation with

secondary antibody horseradish peroxidase (HRP)-linked anti-rabbit antibody, (Cat #7074, Cell Signaling Technology, Beverly, MA, USA) or HRP-linked anti-mouse antibody (Cat #P0260; Dako, Carpinteria, CA, USA), proteins were visualized with enhanced chemiluminescence solution (SuperSignalTM West Pico Plus, Cat #34580, Invitrogen, Oakville, ON, Canada) on Imaging Station 4000MM Pro (Carestream Health, Rochester, NY, USA). Blots were analyzed with Image 1.52 (National Institutes of Health, Bethesda, MD, USA).

III.6 RNA extraction, reverse transcription and real-time semi-quantitative PCR.

III.6.1 Total RNA isolation. Total RNA was isolated from samples using Qiazol reagent (MiroRNEasy kit, Qiagen, Cat #74004, Toronto, ON, Canada), and following the manufacturer's instructions. Total RNA was precipitated from the aqueous phase with chloroform, then washed with 100% ethanol, and finally suspended in a small volume of ribonucleasefree water (Qiagen). The volumes of total RNA solution obtained from each C2C12 well culture was 20uL. Optical density (OD) measurements were made at 260 and 280 nm in order to determine RNA concentration and purity.

III.6.2 Reverse transcription reaction. Reverse transcription (RT) was carried out in a 20ml final volume with 2 ug total RNA for C2C12 well culture. RT was performed using the Cell-to-cDNA II kit (Ambion - Life Technologies, Cat. #1722, Burlington, ON, Canada).

III.6.3 Real-time PCR. cDNA samples were analysed by Taqman real-time quantitative polymerase chain reaction (qPCR) with a Rotor-Gene Q series system (Cat. #9001570, Qiagen, Toronto, ON, Canada) using qPCR master mix (Invitrogen – Life Technologies, Ca. #11743, Burlington, ON, Canada) and Taqman probes targeted to mouse *Thbs1*, *VegfA* and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), followed by quantification using

comparative $\Delta\Delta\text{CT}$ analysis to determine relative mRNA expression. Amplified gene sequences are shown in Table 1. HPRT was used as a housekeeping gene.

III.7 Cycloheximide treatment and half-life measurement. Cycloheximide solution was obtained from Sigma-Aldrich (St-Louis, MO, USA, Cat. #66-81-9, dissolved in DMSO at a concentration of 100mg/mL) and will be used at a concentration of 50ug/mL for 30, 60 or 90 minutes on C2C12 myotubes for THBS-1 and THBS-4 half-life investigations. Half-life will be calculated by Prism8 software using the formula below:

$$t_{1/2} = \frac{\ln(2)}{K}$$

$t_{1/2}$ = half-life

K = rate constant (hours⁻¹, calculated by Prism8)

III.8 Statistical analysis. Statistical analyses were performed using Student's *t* test, 1-way or 2-way ANOVA using Prism8 (GraphPad Software Inc., San Diego, CA, USA). For 1-way and 2-way ANOVA analyses, the Bonferroni multiple comparisons post-hoc test was used. The results were considered to be statistically significant at values of $P \leq 0.05$.

IV. Results

IV.1 Hypoxia induces an angiadaptive switch at the mRNA level in myotubes *in vitro* after 6 hours of exposure.

Differentiated C2C12 myotubes were incubated in a modular incubation chamber with a pre-set gas mixture of 94% N₂, 5% CO₂ and 1% O₂. After each timepoint, cells were immediately lysed, and total RNA was isolated and purified. Samples were analyzed by polymerase chain-reaction (PCR) technique and probed for THBS-1 and VEGF-A transcripts (Figure 1). VEGF-A mRNA was used as a hallmark of hypoxia as it contains a hypoxia response element (HRE) on its promoter region which is activated by HIF-1 α in hypoxic conditions (Yamakawa et al., 2003). As expected, VEGF-A saw a gradual increase

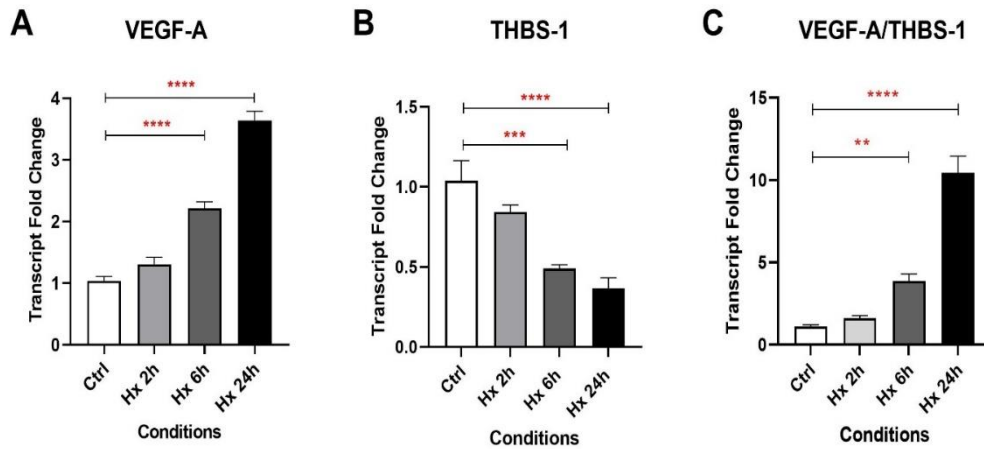


Figure 1. A) Relative expression of VEGF-A mRNA in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Ctrl). **B)** Relative expression of THBS-1 mRNA in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Ctrl). **C)** Ratio of VEGF-A against THBS-1 mRNA average fold change in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Ctrl, 21% O₂). HPRT was used as a housekeeping gene and was not affected by treatment (Figure A1). Data represented as means $\pm$ SEM (A, B, n=5-6 per timepoint and condition; C, n=17-18 per timepoint) and were analyzed by two-way ANOVA with Bonferroni post-hoc comparison. Statistical difference: n.s., not significant versus control or 0h groups; ***, versus controls (P<0.001); ****, versus control or 0h groups (P<0.0001).

with extended exposure to hypoxia, becoming significant compared to controls after 6 hours (h) of exposure: Ctrl vs. Hx 6h (+213%, 1.04 ± 0.16 vs. 2.21 ± 0.26 , $P \leq 0.0001$) (Figure 1A). At 24 h, VEGF-A mRNA was 3.55-fold greater in the hypoxic group versus the control group: Ctrl vs. Hx 24h (+355%, 1.04 ± 0.16 vs. 3.64 ± 0.37 , $P \leq 0.0001$). In contrast, THBS-

1 mRNA expression in C2C12 myotubes is greatly decreased in response to hypoxia (Figure 1B). Hypoxia exposure reduced THBS-1 transcript by 53% after 6 h and 65% after 24 h in comparison to the control group: Ctrl vs. Hx 6h vs. Hx 24h (-53% & - 65%, respectively, 1.04 ± 0.31 vs. 0.49 ± 0.05 vs. 0.37 ± 0.16 , $P \leq 0.001$ and $P \leq 0.0001$, respectively). Hypoxanthine-guanine ribosyltransferase (HPRT) mRNA was used as the housekeeping gene in these experiments (Figure A1). HPRT cycle threshold (CT) values were analyzed by one-way ANOVA with Bonferroni post-hoc test and our housekeeping gene was found to be unaffected by the hypoxic treatment: Ctrl vs. Hx 2h. Hx 6h vs. Hx 24h (26.42 ± 0.32 vs. 26.19 ± 0.34 vs. 25.98 ± 0.40 vs. 26.42 ± 0.52).

IV.2 VEGF-A/THBS-1 mRNA fold-change ratio is greatly increased in differentiated C2C12 myotubes in response to hypoxia.

The angioadaptive balance in myotubes in response to hypoxia was assessed using a VEGF-A/THBS-1 ratio. Fold-change data was used to determine the relative ratio in transcriptional expression of VEGF-A and THBS-1 mRNA in myotubes subjected to 2 h, 6 h or 24 h hypoxic incubation versus controls (Figure 1C). VEGF-A/THBS-1 ratio exponentially increased with extended hypoxia exposure: Ctrl vs. Hx 2h vs. Hx 6h vs. Hx 24h (1.12 ± 0.43 vs. 1.6 ± 0.69 vs. 3.9 ± 1.80 vs. 10.46 ± 24). Interestingly, VEGF-A/THBS-1 ratio in myotubes only became significant after 6 h hypoxia exposure compared to the control group: Ctrl vs. Hx 6h (+346%, 1.12 ± 0.43 vs. 3.88 ± 1.80 , $P \leq 0.01$). These findings coincide both with the significant increase in VEGF-A mRNA, and significant decrease in THBS-1 mRNA seen in myotubes in response to 6 h hypoxia exposure (Figure 1A, B).

IV.3 Total protein concentration is preserved in C2C12 myotubes after 24 h hypoxic exposure, but THBS-1 and THBS-4 intracellular expressions are greatly decreased.

Differentiated C2C12 myotubes were incubated in a modular incubation chamber with a pre-set gas mixture of 94% N₂, 5% CO₂ and 1% O₂. After each timepoint, cells were immediately lysed, and protein lysates were collected. Table 1 shows the average protein concentration in cultured differentiated C2C12 myotubes exposed to 2 h, 6 h or 24 h normobaric hypoxia or normoxia. Protein absorbance measurements were determined by bicinchoninic acid (BCA) assay and no significant difference in protein yields were

Table 1. Differentiated C2C12 myotube protein concentrations in normoxia and normobaric hypoxia

	Total (n)	Protein Concentration (µg/µL)		
		2h	6h	24h
Normoxia	18	3.072 ± 0.11	3.014 ± 0.13	3.279 ± 0.17
Hypoxia	18	3.359 ± 0.12	3.428 ± 0.11	2.808 ± 0.11 ^{γφ}

Table 1. Differentiated C2C12 myotube protein concentrations (µg/µL) after 2-hour (h), 6h or 24h exposure to normobaric hypoxia (1% O₂) or normoxia (21% O₂). C2C12 myoblasts were plated onto 6-well plates at a density of 2.583 x 10⁵ and allowed to grow for 24 hours or until wells were 90% confluent at which point differentiation was induced by media supplementation with 2% horse serum. Cells were allowed to differentiate for 6 days before normobaric hypoxia or normoxia treatments. Cells were collected and lysed immediately after each timepoint and protein concentration was determined by bicinchoninic acid assay. Data represented as means ± SEM (n = 18 per timepoint and condition) and were analyzed by one-way ANOVA with Bonferroni post-hoc test. Statistical difference: γ, versus hypoxia 2h (P<0.05), φ, versus hypoxia 6h (P<0.05).

observed between normoxic and hypoxic groups. Samples were then analysed by Western Blot technique and protein expression was examined by immunoblotting (Figure 2A). Neither THBS-1 nor THBS-4 protein expression showed any change at 2 h and 6 h of hypoxic exposure. At 24 h hypoxic exposure, however, a sharp and drastic decrease in THBS-1 protein expression was observed versus time-matched controls: Nx 24h vs. Hx 24h (-59.1%, 0.83 ± 0.04 vs. 0.35 ± 0.05, P≤0.0001) (Figure 2B). Surprisingly, the same trend was seen in THBS-4 expression at 24 h hypoxic exposure, to a lesser extent: Nx 24h vs. Hx 24h (-52.4%, 0.63 ± 0.13 vs. 0.30 ± 0.02, P≤0.0001). Indeed, the impact of hypoxia on

differentiated C2C12 myotube THBS-1 and THBS-4 intracellular protein expression was strongly positively correlated across all timepoints: $r(16) = 0.91$, $P \leq 0.0001$ (Figure 2C).

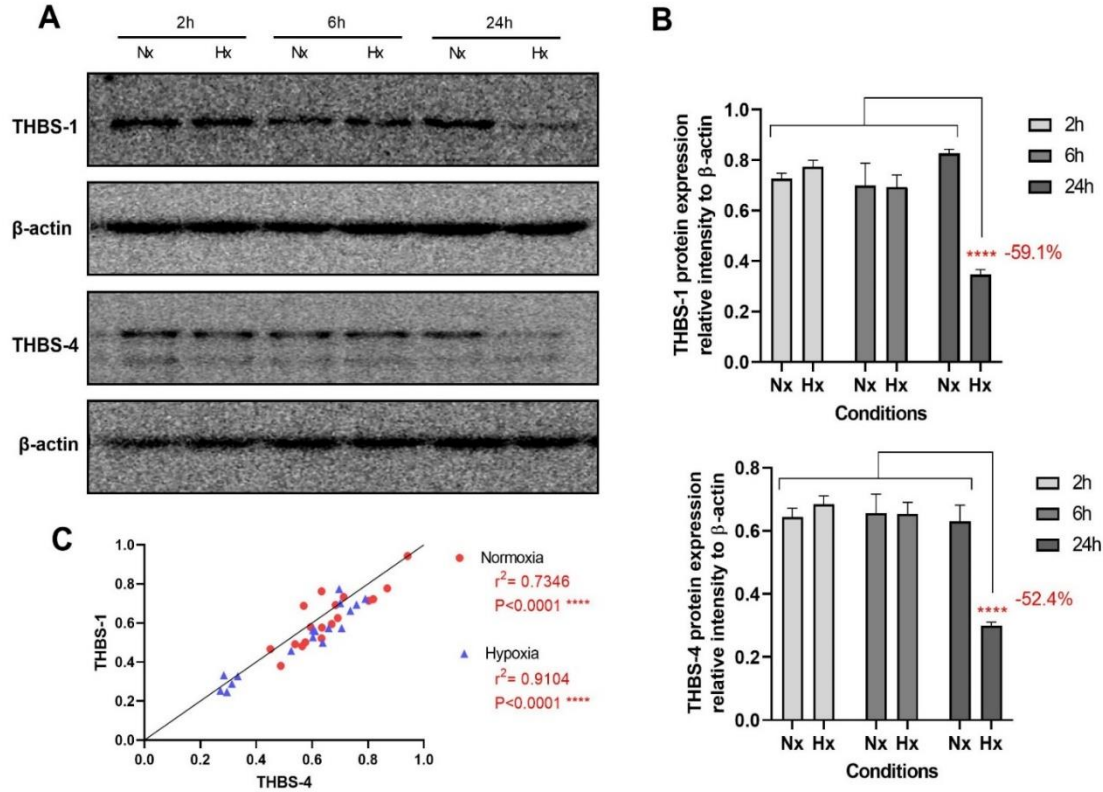


Figure 2. A) Representative immunoblot and densitometry analysis of THBS-1, THBS-4 and β -actin protein expression in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h exposure to normobaric hypoxia (Hx, 1% O_2) or normoxia (Nx, 21% O_2). B) Relative expression of THBS-1 and THBS-4 protein to β -actin protein in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h exposure to normobaric hypoxia (Hx, 1% O_2) or normoxia (21% O_2). C) Correlation between THBS-1 and THBS-4 relative protein expression in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h exposure to normobaric hypoxia (1% O_2) or normoxia (21% O_2). β -actin was used as a loading control. Data represented as means $\pm$ SEM (n=5-6 per timepoint and condition) and were analyzed by two-way ANOVA with Bonferroni post-hoc comparison (B) or correlation analysis (C). Statistical difference: ****, versus all other conditions ($P < 0.0001$).

IV.4 Hypoxia reduces secreted THBS-1 protein in the conditioned media of C2C12 myotubes, but not THBS-4.

Differentiated C2C12 myotubes were incubated in a modular incubation chamber with a pre-set gas mixture of 94% N_2 , 5% CO_2 and 1% O_2 for 24 h. After treatment, conditioned cell media was collected and concentrated. Samples were analysed by Western Blot technique and protein secretion was examined by immunoblotting (Figure 3A).

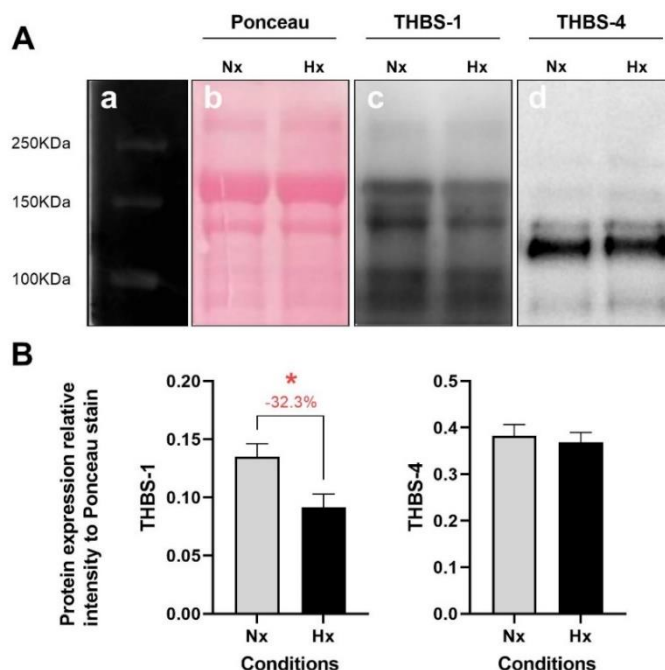


Figure 3. A) Representative images corresponding to immunoblots and densitometry analysis of differentiated C2C12 myotube secreted THBS-1 and THBS-4 protein from concentrated conditioned media in response to 24-hour (h) exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂); **a**, molecular weight ladder; **b**, Ponceau stain of membrane used as a loading control; **c**, immunoblot and densitometry analysis of secreted THBS-1 protein from C2C12 concentrated conditioned media; **d**, immunoblot and densitometry analysis of secreted THBS-4 protein from C2C12 concentrated conditioned media. **B)** Differentiated C2C12 myotube secreted THBS-1 and THBS-4 protein in concentrated conditioned media in response to 24h exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂). Ponceau stain was used as a loading control. Data represented as means $\pm$ SEM (n=5-6 per timepoint and condition) and were analyzed by two-tailed T-test. Statistical difference: *, (P<0.05).

Secreted THBS-1 protein was moderately decreased after 24 h hypoxic exposure, compared to time-matched controls: Nx vs. Hx (-32.3%, 0.14 ± 0.01 vs. 0.09 ± 0.01 , $P \leq 0.05$) (Figure 3B). Interestingly, despite the drastic decrease observed in the intracellular expression of THBS-4 protein in hypoxic myotubes, secreted THBS-4 protein in the conditioned media was unaffected by hypoxia: Nx vs. Hx (0.38 ± 0.02 vs. 0.37 ± 0.02 , n.s.). In contrast to the intracellular protein findings which were found to be strongly associated, hypoxia differentially impacts these proteins outside the cell.

IV.5 90-minute exposure to hypoxia impacts THBS-1 and THBS-4 protein stability, and greatly increases THBS-4 half-life.

To assess protein stability in myotubes under normoxic or hypoxic conditions, cells were treated with 50 μ g/ μ L cycloheximide (CHX), a protein synthesis inhibitor, dissolved

in dimethyl sulfoxide (DMSO) solution. Cells were then placed under 1% O₂ conditions similar to previous experiments and a cycloheximide chase assay was conducted. After each timepoint, cells were immediately lysed, and protein lysates were collected. Samples were analysed by Western Blot technique and protein expression was examined by immunoblotting (Figure 4A). CHX treatment was effective in blocking THBS-1 protein synthesis, made apparent by the gradual decrease seen in THBS-1 protein expression in the CHX groups across all timepoints and in both conditions: Nx -CHX vs. Nx +CHX and Hx -CHX vs. Hx +CHX ($P \leq 0.0001$ & $P \leq 0.0001$, respectively) (Figure 4B). The same effect was observed in THBS-4 protein synthesis, both in normoxic and hypoxic conditions: Nx -CHX vs. Nx +CHX and Hx -CHX vs. Hx +CHX ($P \leq 0.0001$ & $P \leq 0.0001$, respectively)

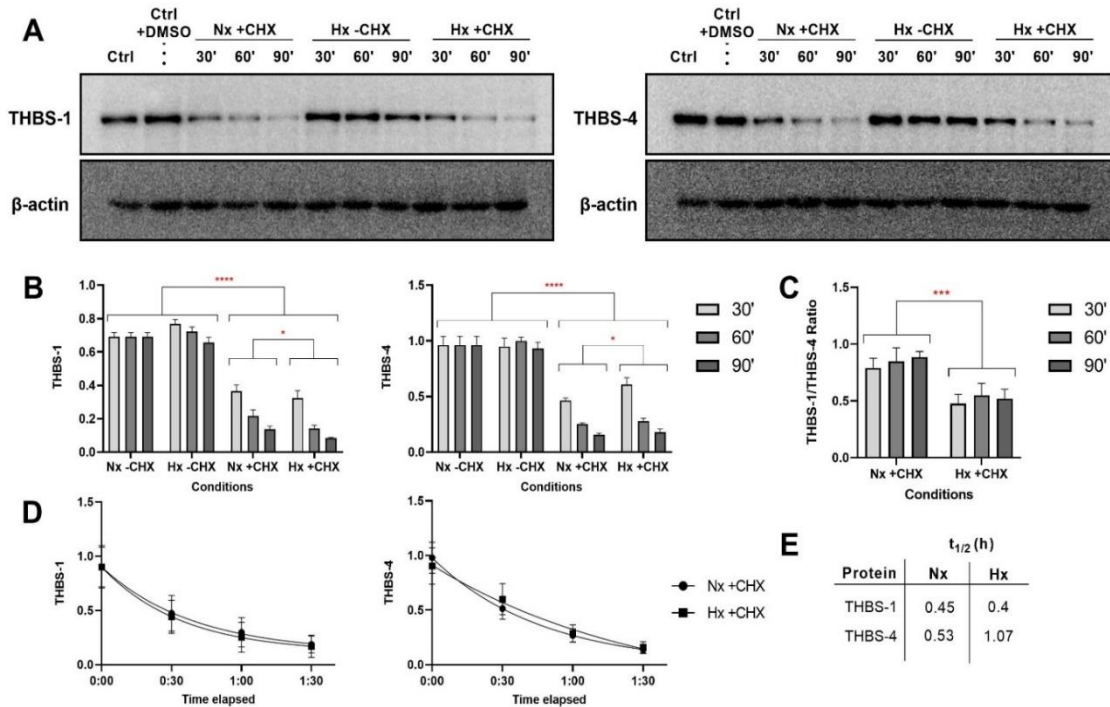


Figure 4. A) Representative immunoblot and densitometry analysis of THBS-1, THBS-4 and β -actin protein expression in differentiated C2C12 myotubes in response to 30-minute ('), 60' or 90' exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂) with (+) or without (-) 50 μ g/ μ L cycloheximide (CHX) treatment. **B)** Relative expression of THBS-1 and THBS-4 protein to β -actin protein in differentiated C2C12 myotubes in response to 30-minute ('), 60' or 90' exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂) with (+) or without (-) 50 μ g/ μ L cycloheximide (CHX) treatment. **C)** Relative expression ratio of THBS-1 protein against THBS-4 protein relative to β -actin protein in differentiated C2C12 myotubes treated with 50 μ g/ μ L cycloheximide (CHX) in response to 30-minute ('), 60' or 90' exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂). **D)** Relative change in expression of THBS-1 and THBS-4 protein to β -actin protein in differentiated C2C12 myotubes in response to 0- minute ('), 30', 60' or 90' exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂). **E)** Half-life ($t_{1/2}$) measurements of THBS-1 and THBS-4 protein in differentiated C2C12 myotubes in response to 30-minute ('), 60' or 90' exposure to normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂) reported in hours (h). β -actin was used as a loading control. 2 μ L DMSO was used as a negative control. Data represented as means $\pm$ SEM (B, C n=5-6 or D, E, n = 11-12 per timepoint and condition) and were analyzed by two-way ANOVA with Bonferroni post-hoc comparison. Statistical difference: *, versus Nx +CHX conditions ($P < 0.05$); ***, versus Nx+CHX conditions ($P < 0.001$); ****, versus Nx -CHX and Hx -CHX conditions ($P < 0.0001$)).

(Figure 4B). 90-minute hypoxic exposure was found to moderately reduce THBS-1 protein stability compared to normoxic controls: Nx +CHX vs. Hx +CHX (-24%, 0.24 vs. 0.18, $F(1,30) = 5.384$, $P \leq 0.05$) (Figure 4B). Hypoxia also altered THBS-4 protein stability compared to normoxic controls, but oppositely to THBS-1 protein, THBS-4 protein stability was found to be moderately increased: Nx +CHX vs. Hx +CHX (+22%, 0.29 vs. 0.36, $F(1,30) = 6.098$, $P \leq 0.05$). To support these findings, THBS-1/THBS-4 protein ratios were taken at all timepoints and were analyzed by two-way ANOVA with Bonferroni post-hoc test. The ratios were significantly lower in the hypoxic conditions thus confirming the two proteins were being differentially impacted by the treatment: Nx +CHX groups vs. Hx +CHX groups (-39%, 0.84 vs. 0.52, $F(1,29) = 19.39$, $P \leq 0.001$) (Figure 4C).

Protein half-life ($t_{1/2}$) was determined by cycloheximide chase assay for both THBS-1 and THBS-4 proteins in differentiated C2C12 myotubes (Figure 4D & E). The calculated $t_{1/2}$ of THBS-1 protein in normoxic and hypoxic conditions were 0.45 h and 0.40 h, respectively. In contrast, the calculated $t_{1/2}$ of THBS-4 was increased 2-fold in hypoxia compared to normoxic controls: Nx vs. Hx (0.53h vs 1.07h, respectively).

V. Discussion

Skeletal muscle angioadaptation is a tightly regulated process involving a balance of pro- and antiangiogenic factors within the muscle tissue. However, the involvement of myofibers, the major cell population in skeletal muscle, in the angioadaptive balance has rarely been studied in the context of hypoxia. Differentiated C2C12 myotubes are a well-established model for studying contractile myofibers *in vitro* and have been shown to respond to hypoxia similarly to myofibers *in vivo* (Yadav et al., 2014). Despite this, no study has yet investigated the impact of hypoxia on the expression and secretion of THBS-1 protein, the key antiangiogenic factor, in myofibers. More so, recent studies have suggested a proangiogenic role for THBS-4 in cardiac muscle, Lung EC and tumours (Frolova et al., 2014; Muppala et al., 2015). Yet, whether it contributes to angioadaptation in skeletal muscle, and whether its expression and secretion is impacted by hypoxia, is unknown. In the current study, we show that hypoxia represses THBS-1 at the level of its mRNA, protein expression and secretion in C2C12 myotubes, and that hypoxia differentially regulates THBS-1 and THBS-4 at the level of their protein stability and secretion in these cells.

Yadav et al. (2014) have previously shown that a hypoxia-mimetic drug, DMOG, represses PGC-1 β expression in C2C12 myotubes and that THBS-1 transcription is moderately repressed in PGC-1 $\beta^{-/-}$ muscle. Here, we provide direct evidence that THBS-1 transcription is drastically decreased in C2C12 myotubes in response to ambient hypoxia (Figure 1B), confirming the physiological effect of hypoxia on its transcription. Furthermore, VEGF-A transcription was drastically increased in the same conditions,

validating our hypoxic model (Figure 1A). Interestingly, the temporal fold-change in the expression of both genes coincided at the 6 h timepoint of our treatment and were nearly identically, but inversely, impacted by hypoxia. More accurately, in response to 6 h exposure to hypoxia, THBS-1 transcription in C2C12 myotubes was roughly halved while VEGF-A transcription was roughly doubled. Accordingly, the VEGF/THBS-1 ratio reaches significance at the 6 h mark (Figure 1C).

The upregulation of VEGF-A transcript in response to 6 h exposure to hypoxia in our experiments is in agreement with previous reports of the temporal response of VEGF-A mRNA observed in the muscle of mice subjected to 1 h of acute exercise on a treadmill (Breen et al., 1996; Kivela et al., 2008). Interestingly, this could support the notion that hypoxia is a primary component of exercise stimulus, and that myotube-derived VEGF-A is the major contributor in previous findings of the whole-muscle VEGF-A response to exercise. Moreover, the concurrent reduction in myotube THBS-1 transcript suggests that hypoxia acts as a dual regulator of skeletal muscle angioadaptation, impacting both the pro- and antiangiogenic sides of the balance. While these results are not surprising, they confirm that myotubes inherently respond to hypoxic stress, support the findings from Yadav et al., (2014), Breen et al., (1996) and Kivela et al., (2008) and provide novel evidence for the involvement of myotubes in shifting the angioadaptive balance in skeletal muscle in response to hypoxia.

For our protein investigations, we had to confirm that the hypoxic treatment did not impact total protein concentration within differentiated C2C12 myotubes. Total cell lysates from our normoxic and hypoxic samples were examined by BCA assay, and the results were analyzed by two-way ANOVA with Bonferroni post-hoc comparison (Table 1). The

analysis found no significant difference between the normoxic groups and hypoxic groups. As well, media starvation effects were investigated to ensure starvation was not a confounding variable in our treatment length (Figure A2). Indeed, no observable change in THBS-1 protein expression was observed in differentiated C2C12 myotube subjected to 48 h without media replenishment.

For angioadaptation to occur at the level of the muscle in response to a proangiogenic stimulus such as exercise or hypoxia, THBS-1 protein expression must be repressed (Biot & Olfert, 2011; Olfert, 2016). While this response has been observed at the whole muscle level, the expression of THBS-1 protein in myotubes, specifically, has never been examined. The current study reveals that C2C12 myotube intracellular THBS-1 expression exhibits a sharp and drastic decrease after 24 h hypoxic exposure (Figure 2B). This finding may, in part, be explained by the repression of THBS-1 transcript in response to hypoxia. However, proteasomal degradation may also contribute to the abolishment of THBS-1 protein expression in C2C12 myotubes in response to hypoxia and would be a good target for future investigations.

Recent findings from Frolova et al. (2014) and Muppala et al. (2015, 2017) have placed particular interest on THBS-4 in the angioadaptive balance, as their studies provide evidence that THBS-4 may have potential proangiogenic properties. We therefore chose to examine the impact of hypoxia on myotube THBS-4 expression to assess whether it may play a role in skeletal muscle angioadaptation and hypothesized that hypoxia would inversely impact its expression compared to THBS-1. To our surprise, THBS-4 intracellular protein expression in differentiated C2C12 myotubes exhibited a strong decrease in response to 24 h hypoxia exposure, similarly to THBS-1 (52.4% vs 59.1%, respectively)

(Figure 2B). This response was very strongly correlated with the decrease in THBS-1 protein expression (Figure 2C), suggesting that hypoxia impacts THBS-1 and THBS-4 protein expression in the myofiber through a common mechanism. Indeed, this data does not support THBS-4 as a proangiogenic factor in myotubes *per se*, though it is in agreement with reports which suggest THBS-4 expression may possess a tight relationship with oxygen in muscle (Frolova et al., 2014). The study from Frolova et al. (2014) found that the expression pattern of THBS-4 in muscle was detected in close proximity to type I fibers, but not type type IIa or type IIb fibers, as well as skeletal muscle endothelial cells. Accordingly, the low oxygen availability in our treatment model downregulated THBS-4 protein expression in the myotubes suggesting its expression in muscle is oxygen-dependent. More research must be conducted on the mechanisms which might repress the expression of THBS-4 protein in myotubes to confirm its involvement in skeletal muscle angioadaptation.

THBS-1 and THBS-4 are secreted glycoproteins, thus their functions are primarily reserved to the ECM. Secreted THBS-1 and THBS-4 proteins were measured in the conditioned media of C2C12 myotubes exposed to 24 h normoxia or normobaric hypoxia (Figure 4). In line with the decreases in THBS-1 expression at the mRNA and intracellular protein levels, THBS-1 protein in the conditioned media was found to be moderately decreased in the hypoxic group (Figure 3B). To our knowledge, this is the first evidence that THBS-1 protein is secreted from differentiated C2C12 myotubes and that myotube-derived secreted THBS-1 expression is decreased in hypoxia. These results could suggest a mechanism by which extracellular THBS-1 protein is endocytosed by C2C12 myotube cultures in hypoxia and might occur through heparin sulfate proteoglycans (HSP) and LRP1

on the plasma membrane of these cells (Godyna et al., 1995; Mikhaelenko et al., 1995; Feitsma et al., 2000)

In contrast, THBS-4 protein in the conditioned media of hypoxic C2C12 myotubes was identical to the normoxic group despite exhibiting a decrease similar to THBS-1 intracellularly in the myotubes (Figure 3B). This suggests that THBS-4 protein is differentially impacted in- and outside of the myofiber in response to hypoxia, and that THBS-1 and THBS-4 are differentially impacted by 24 h hypoxic exposure in the ECM. In this sense, it is possible that THBS-1 and THBS-4 follow a common hypoxia-induced degradation pathway intracellularly, but not extracellularly.

Lastly, we sought to investigate the protein stability of both THBS-1 and THBS-4. All proteins are subject to half-life as protein turnover is a critical part of cell survival. Thrombospondins are known to have rapid turnover (Murphy-Ullrich et al., 1987), yet no previous study has assessed THBS-1 nor THBS-4 half-life ($t_{1/2}$) in differentiated myotubes. Here, we report that the $t_{1/2}$ of THBS-1 and THBS-4 in differentiated C2C12 myotubes are roughly 0.45 h and 0.53 h, respectively, in normoxic conditions (Figure 4E). Interestingly, however, while hypoxia did not greatly alter the $t_{1/2}$ of THBS-1 protein, THBS-4 protein saw its $t_{1/2}$ increase 2-fold in hypoxic conditions (0.53h vs. 1.07h) (Figure 4D). Contrary to the THBS-4 protein expression data, these results suggest hypoxia may stabilize THBS-4 protein in differentiated C2C12 myotubes, at least transiently. Indeed, the THBS-1/THBS-4 protein ratio in the Hx +CHX conditions is greatly reduced in comparison to the Nx +CHX conditions, suggesting that THBS-4 protein stability is preferentially preserved compared to THBS-1 in C2C12 myotubes in response to hypoxic exposure (Figure 4C). Given the decrease in THBS-4 protein expression in response to 24 h of hypoxic exposure (Figure

2B), one explanation may be that THBS-4 protein degradation is delayed in hypoxia, but not inhibited. This would suggest that THBS-4 serves an adaptive function in the early stages of myotube response to hypoxia. Taken together, these findings elucidate the differential impact of hypoxia on THBS-1 and THBS-4 protein stability in myotubes under hypoxia and support the possibility of differential functions for the two proteins in the early stages of the angiadaptive response in muscle under hypoxia. Though, investigations regarding the impact of hypoxia on differentiated myotube THBS-4 mRNA expression should be conducted to test this assumption.

Conclusion

In the current study, we have shown that ambient hypoxia impacts differentiated C2C12 myotube THBS-1 at 1) the transcriptional level, 2) at the level of its intracellular protein and its stability, and 3) its secretion. Hypoxia induced an angiadaptive switch at the transcriptional level whereby VEGF-A mRNA increased by a factor of 2 while THBS-1 mRNA decreased by a factor of 2 after 6 h hypoxic exposure. The VEGF-A/THBS-1 mRNA ratio was greatly increased at 6 h and depicted a dual-regulatory effect of hypoxia on the angiadaptive response in differentiated myotubes. Moreover, it appears that this temporal response is in line with the temporal response seen in acute exercise and thus elucidates the contribution of myotube-derived angiadaptive factors to the whole-muscle angiadaptive response to exercise. 24 h hypoxia exposure was found to decrease THBS-1 and THBS-4 intracellular protein expression to similar degrees, however in the short-term, seemed to preferentially preserve THBS-4 protein stability over THBS-1. The $t_{1/2}$ of both THBS-1 and THBS-4 proteins were reported in myotubes for the first time, being 0.45 h and 0.53 h in normoxia, respectively. In hypoxia, the $t_{1/2}$ of THBS-4 protein was doubled

while THBS-1 $t_{1/2}$ was virtually unchanged. 24 h hypoxic exposure also differentially impacted secreted THBS-1 and THBS-4 protein concentrations. More accurately, THBS-1 secreted protein was moderately decreased in the ECM while THBS-4 protein concentration was identical to normoxic controls, suggesting that 24 h exposure to ambient hypoxia similarly impacts the intracellular expression of these homologues in myotubes but differentially impacts their expression in the extracellular space. Taken together, these findings provide new evidence for the responsiveness of myotubes to hypoxic stress and their contribution to the angioadaptive balance in skeletal muscle, as well as insights on the differential expression of THBS-1 and THBS-4 in hypoxic myotubes and their secretome.

VI. Limitations

1. A major limitation of this study is the effect of reoxygenation. In the time between removing the samples from hypoxic incubation and collecting the cell lysates/conditioned media samples, it is likely that a slight degree of reoxygenation may occur and generate added variability within the results of various independent experiments.

VII. Future Research

1. To investigate the impact of ambient hypoxia on the transcription of THBS-4 in C2C12 myotubes in order to build a greater understanding of the differential impact it has on THBS-1 and THBS-4 in myotubes.
2. To determine whether hypoxic myotube conditioned media has a greater impact on endothelial cell migration and proliferation *in vitro* versus the conditioned media of normoxic controls.
3. To determine the impact of exercise on the expression of THBS-4 protein and transcription in skeletal muscle.

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Appendices

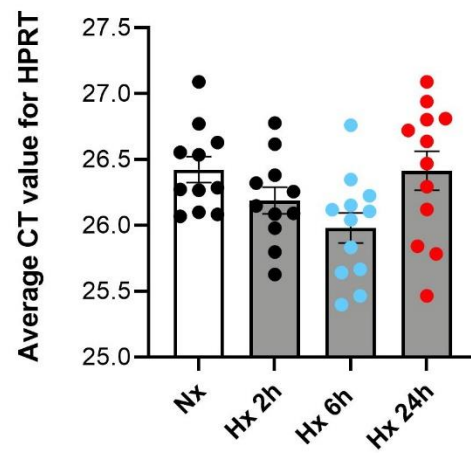


Figure A1: Average CT values for HPRT expression in differentiated C2C12 myotubes in response to 2-hour (h), 6h or 24h normobaric hypoxia (Hx, 1% O₂) or normoxia (Nx, 21% O₂). HPRT was used as a housekeeping gene and was found to not be impacted by hypoxia exposure. Data represented as means $\pm$ SEM (n = 11-12 per condition) and were analyzed by one-way ANOVA with bonferroni post-hoc comparison. Statistical difference: *, (P<0.05) versus Nx.

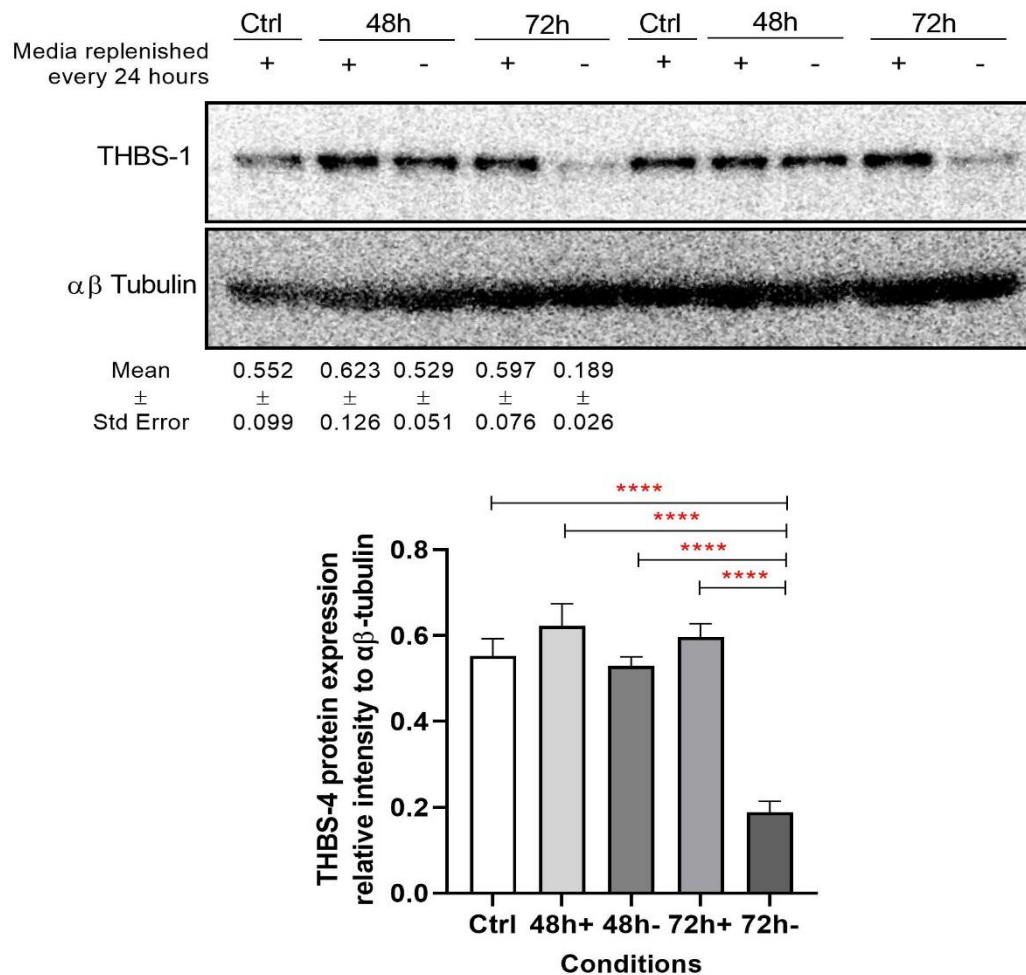


Figure A2. THBS-1 protein expression relative to $\alpha\beta$ -tubulin in differentiated C2C12 myotubes in response to 48-hours (h) or 72h with (+) or without (-) media replenishment every 24 hours. Data represented as means $\pm$ SEM (n=5-6 per timepoint and condition) and were analyzed by one-way ANOVA with Bonferroni post-hoc test. Statistical difference: ****, (P<0.0001) versus 72h-.