INVESTIGATING THE IMPACT OF ENVIRONMENTAL STRESSORS ON SKELETAL MUSCLE ANGIO-ADAPTATION

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Abstract

Skeletal muscle angio-adaptation refers to coordinated molecular and cellular events that regulate the expansion (angiogenesis) or regression of the muscle capillary network. The influence of environmental stressors (hypoxia, cold-stress) on skeletal muscle angio-adaptive responses are less understood in comparison to exercise-training or diseased states. This thesis addresses the influence of hypoxia and cold-stress on skeletal muscle angio-adaptation through a literature review and original research, respectively. After reviewing the literature, ambient hypoxia alone may be insufficient in improving muscle capillarization, however ambient hypoxia superimposed with exercise enhances the angiogenic response of exercise-training. *In-vitro*, the effect of cold-stress with respect to the angiogenic relationship between the myocyte and endothelial cell was investigated. Cold-stress reduced the secretion of angiogenic molecules from the myocyte. Furthermore, cold-stress abrogated endothelial cells proliferation and attenuated VEGFR2 activation. Endothelial cells pre-conditioned to cold-stress and challenged for migration in re-warming conditions however, exhibited an enhanced migratory responsiveness.

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List of Abbreviations

ATF6	**Activating transcription factor 6**
ADAMTS-1	**A disintegrin and metalloproteinase with thrombospondin motif**
ANOVA	**Analysis of Variance**
CD	**Capillary density**
C/F	**Capillary-to-fiber ratio**
CD26	**Cluster of differentiation 26**
CD31	**Cluster of differentiation 31**
CD36	**Cluster of differentiation 36**
CD47	**Cluster of differentiation 47**
CXCl1	**C-X-C Motif chemokine ligand 1**
CXCl10	**C-X-C Motif chemokine ligand 10**
CXCl12	**C-X-C Motif chemokine ligand 12**
CXCl16	**C-X-C Motif chemokine ligand 16**
CX3CL1	**Fractalkine**
Cyr61	**Cysteine-rich angiogenic inducer 61**
Dll4	**Delta-like 4**
DMEM	**Dulbecco's modified eagle medium**
ECGS	**Endothelial cell growth supplement**
ECL	**Enhanced luminol-based chemiluminescence**
EDTA	**Ethylenediaminetetraacetic acid**
EGF	**Epidermal growth factor**
ELISA	**Enzyme-linked immunosorbent assay**
EPO	**Erythropoietin**
ER-stress	**Endoplasmic reticulum stress**
EMI	**Ex-vivo muscle incubation**
FBS	**Fetal bovine serum**
FGFs	**Fibroblast growth factors**
aFGF	**Acidic fibroblast growth factor**
bFGF	**Basic fibroblast growth factor**

FGF7	**Keratinocyte growth factor**
FIH	**Factor inhibiting HIF-1**
FiO_2	**Fraction of inspired oxygen**
Fyn	**Proto-oncogene tyrosine-protein kinase Fyn**
GM-CSF	**Granulocyte-macrophage colony-stimulating factor**
Grp78	**Glucose regulated protein 78**
HB-EGF	**Heparin binding epidermal growth factor**
HGF	**Hepatocyte growth factor**
HIF-1	**Hypoxia inducible factor 1**
HIF-1α	**HIF-1 alpha**
HRE	**Hypoxia responsive element**
HRP	**Horseradish peroxidase**
HPRT	**Hypoxanthine-guanine phosphoribosyltransferase**
HuR	**Human antigen R**
IGFBP-1	**Insulin like growth factor binding protein 1**
IGFBP-2	**Insulin like growth factor binding protein 2**
IGFBP-3	**Insulin like growth factor binding protein 3**
IGFBP-10	**Insulin like growth factor binding protein 10**
IL-1a	**Interleukin 1 alpha**
IL-8	**Interleukin 8**
IL-10	**Interleukin 10**
IP-10	**Interferon gamma-induced protein 10**
KC	**Keratinocyte-derived chemokine**
Ki-67	**Marker of proliferation ki-67**
LHLT	**Live high train low**
LHTLLH	**Live high train low and high**
LLTL	**Live low train low**
MAPK/ERK	**Mitogen-activated protein kinases/extracellular signal-regulated kinase**
MCP-1	**Monocyte chemoattractant protein 1**

MIP-1a	**Macrophage inflammatory protein 1 alpha**
MMP-3	**Matrix metalloproteinase 3**
MMP-8	**Matrix metalloproteinase 8**
MMP-9	**Matrix metalloproteinase 9**
mRNA	**Messenger ribonucleic acid**
mVEGF-A165	**Murine recombinant VEGFA-165**
MWCO	**Molecular weight cut off**
NaCl	**Sodium chloride**
NaF	**Sodium fluoride**
Na3VO4	**Sodium orthovanadate**
NOV	**Nephroblastoma overexpressed**
ORP-150	**Oxygen regulated protein 150**
PAI-1	**Plasminogen activator inhibitor 1**
PBS	**Phosphate buffered saline**
PD-ECGF	**Platelet derived endothelial cell growth factor**
PDGFs	**Platelet derived growth factors**
PDGF-AA	**Platelet derived growth factor AA**
PDGF-AB	**Platelet derived growth factor AB**
PDGF-BB	**Platelet derived growth factor BB**
PEDF	**Pigment epithelium derived growth factor**
PHD 1-3	**Prolyl hydroxylase 1-3**
PlGF-2	**Placental growth factor 2**
PMSF	**Phenylmethylsulfonyl fluoride**
PO_2	**Partial pressure of oxygen**
p-VEGFR2-Y1175	**Phosphorylated VEGFR2 Tyrosine 1175**
RBM3	**RNA-binding motif 3**
SDS	**Sodium dodecyl sulfate**
SDS-PAGE	**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis**
SDF-1	**Stromal derived factor 1**
SEM	**Standard error of the mean**

SHP-1	**Src homology 2 domain-containing protein tyrosine phosphatase 1**
SIRT6	**Sirtuin 6**
SMECs	**Skeletal muscle endothelial cells**
TGF-β	**Transforming growth factor beta**
THBS1	**Thrombospondin-1**
THBS2	**Thrombospondin-2**
THBS4	**Thrombospondin-4**
TIMP-1	**Tissue inhibitor of metalloproteinase 1**
TIMP-4	**Tissue inhibitor of metalloproteinase 4**
VEGF-A	**Vascular endothelial growth factor-A**
VEGF-B	**Vascular endothelial growth factor-B**
VEGFR1	**VEGF-receptor 1 (Flt-1)**
VEGFR2	**VEGF-receptor 2 (KDR/Flk-1)**
VEGFR3	**VEGF-receptor 3 (Flt-4)**

List of Publications

STUDY 1

Review article.

Lemieux, P., Birot, O., 2021. Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story. Front. Physiol. 12, 735557. https://doi.org/10.3389/fphys.2021.735557

STUDY 2

Research manuscript.

Lemieux, P., Roudier, E., Birot, O., 2022. Angiogenic move or angiostatic freeze? Evaluating the angio-adaptive impact of acute cold stress exposure on cultured murine skeletal muscle myotubes and endothelial cells. Manuscript in preparation for submission to *Frontiers in Physiology*.

Conference Abstract.

Lemieux, P., Roudier, E., Birot, O., 2022. Move or freeze? Elucidating the angiogenic relationship between mouse skeletal muscle endothelial cells and differentiated C2C12 myotubes. 18th International Biochemistry of Exercise Conference (IBEC), Toronto, Canada. May 25-28, 2022.

1. LITERATURE REVIEW

1.1. Functions and plasticity of skeletal muscle tissue.

Skeletal muscle is a remarkably plastic tissue representing one of the largest organs of the human body by compromising for roughly 40% of total body mass (Frontera and Ochala, 2015). In response to various physiological, pathological, and environmental stressors, skeletal muscle myofibers can adapt through alterations in size, contractile phenotype, and metabolic profile (Booth and Thomason, 1991; Frontera and Ochala, 2015). Functionally, the skeletal muscle is a multi-faceted organ involved in locomotion, force production, metabolism, hormone production, and thermoregulation (Frontera and Ochala, 2015; Fuller-Jackson and Henry, 2018; Hoffmann and Weigert, 2017).

1.2. Skeletal muscle capillaries: an important determinant of muscle function.

Capillaries are the smallest blood vessels found in the body (Adair and Montani, 2010). The importance of skeletal muscle capillaries is well established in ensuring optimal blood flow to muscle tissue (Krogh, 1919a, 1919b, 1919c). Indeed, the capillary-to-myofiber interface serves as a direct conduit for the interaction between the circulation and the muscle cell. By allowing for the exchange of oxygen and carbon dioxide, facilitating the delivery of substrates and removal of metabolites, and promoting the circulation of muscle-produced myokines and heat, the capillary microvasculature supports the skeletal muscle in performing it's effector functions of locomotion, metabolism, hormonal production, and thermoregulation. The density of capillaries within a given area of muscle tissue can therefore be seen as a key determinant of skeletal muscle function (Hoppeler, and Kayar, 1988; Hudlicka, 2011; Hudlicka et al., 1987; Lemieux and Birot, 2021; Liu et al., 2012; Mathieu-Costello, 1994). For example, correlations are observed between muscle capillary density (CD) and muscle fatigue resistance (Ballak et al., 2016; Egginton et al., 1998), O_2 conductance (Howlett et al., 2003), insulin sensitivity and glucose tolerance (Prior et al., 2015; Snijders et al., 2017), and exercise capacity (Olfert et al., 2009).

Like skeletal muscle myofibers, muscle capillaries are extremely plastic and undergo remodelling in response to various physiological, pathological, and environmental stressors. Muscle capillaries can compensate for an increased, or decreased blood flow demand, through the growth of novel capillaries from existing blood vessels (angiogenesis) or reducing the number of

existing capillaries (capillary rarefaction), respectively. For example, the physiological stressor of repeated physical exercise, such as endurance training, stimulates the formation of novel muscle capillaries (angiogenesis) (Aiken et al., 2016; Andersen, 1975; Andersen and Henriksson, 1977; Brodal et al., 1977; Roudier et al., 2012; Slopack et al., 2014). Conversely, reduced physical activity (hypokinesia) and various diseased states (e.g., peripheral artery disease, diabetes, and chronic obstructive pulmonary disorder) result in capillary rarefaction (Aiken et al., 2019; Gouzi et al., 2013; Roudier et al., 2013, 2010).

1.3. Assessing skeletal muscle capillarization.

Given the dynamic nature muscle capillaries, consistent means to depict the extent of capillary growth, or regression quantitatively is needed. The histological measurements of capillary density (CD) and capillary-to-fiber ratio (C/F) provide an accurate way to depict the size of a muscle capillary network (Adair and Montani, 2010; Hudlicka et al., 1992). CD refers to the number of capillaries per surface area of tissue, as such any change in CD will reflect the number of capillaries supplying a given surface area of muscle tissue (Adair and Montani, 2010; Hudlicka et al., 1992). For example, during endurance training, the growth of novel capillaries, angiogenesis, will be evidenced by an improved muscle CD (Andersen, 1975; Andersen and Henriksson, 1977). An improved muscle CD is therefore indicative of a greater muscle capillary supply. Conversely, during capillary regression, a reduced muscle capillary supply is exhibited by a reduced muscle CD. However, there are instances in which alterations in muscle CD occur independently of the growth or reduction of muscle capillary network. For example, during myofiber atrophy a reduction in the size of myofiber-cross sectional area leads to a greater number of existing capillaries occupying a similar surface area of tissue improving muscle CD (Degens et al., 2008; Paudyal et al., 2018). Furthermore, capillary growth is not always reflected by changes in CD. For instance, during myofiber hypertrophy, larger myofiber size leads to the compensatory growth of novel capillaries to maintain muscle CD, this being reflected by an improved muscle C/F ratio (Degens et al., 1992). While CD represents the number of capillaries per surface area of tissue, C/F ratio represents the number of capillaries per individual muscle fiber. Therefore, in comparison to CD, C/F ratio represents an accurate indicator of true muscle angiogenesis in that it is reflective of novel capillary formation.

While these histological parameters describe the growth or regression of the muscle capillary network, they are measurements reflecting tissular outcomes. Thus, they fail to encapsulate preceding molecular and cellular events which influence capillary remodelling leading up to eventual tissular adaptation. As such, the concept of "angio-adaptation" has been proposed to provide an integrative view to skeletal muscle capillary remodelling in response to physiological, pathological, and environmental stressors (Lemieux and Birot, 2021; Olfert and Birot, 2011).

1.4. The concept of skeletal muscle angio-adaptation.

Angio-adaptation refers to the dynamic and multi-stepped processes that occur at molecular, and cellular levels, which regulate the tissular adaptations of the growth, stabilization, or regression of muscle capillaries (Figure 1). At the molecular level, skeletal muscle angio-adaptation is regulated by an intricate balance of a plethora of pro- and anti- angiogenic molecules (Egginton and Birot, 2014; Hellsten and Hoier, 2014; Lemieux and Birot, 2021; Olfert, 2016; Olfert et al., 2015; Olfert and Birot, 2011). At the cellular level, angio-adaptation can require the ability of skeletal muscle endothelial cells to proliferate, migrate, and assemble into vascular tubes in the context of angiogenesis. Conversely, angio-adaptation can also involve endothelial cell apoptosis in the context of capillary regression (Gavin, 2009; Hoier and Hellsten, 2014; Hudlicka et al., 1992). Cell-to-cell interactions also play a large role in regulating angio-adaptive processes. For example, the myofiber actively produces and releases angiogenic molecules to act in a paracrine manner on neighbouring endothelial cells influencing their proliferative and migratory responses (Birot et al., 2004; Hoier and Hellsten, 2014; Olfert et al., 2010, 2009). Finally, skeletal muscle angio-adaptation is evidenced at the tissular level through the aforementioned histological parameters of C/F ratio and CD. In the following sections, molecular expression, and cellular behaviour relevant to skeletal muscle angio-adaption will be discussed.

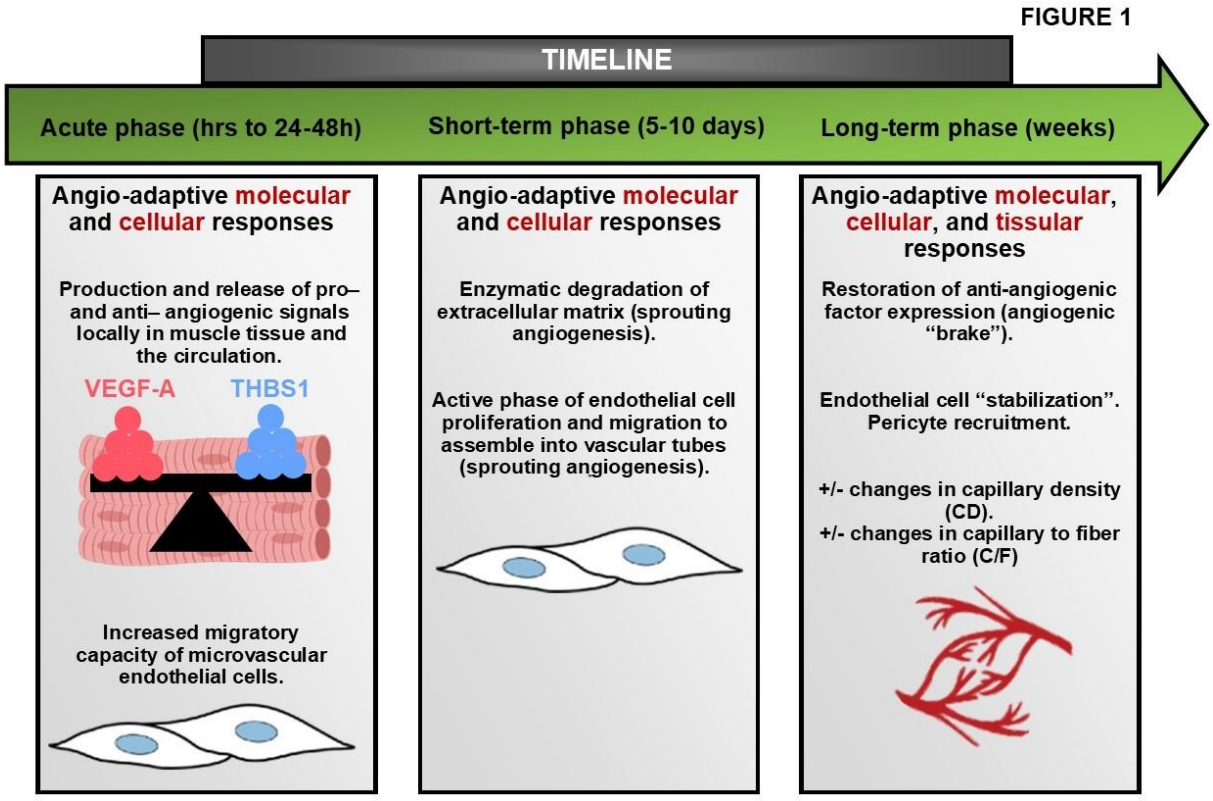

Figure 1. Schematic representation of skeletal muscle angio-adaptation.

Skeletal muscle angio-adaptation refers to dynamic and coordinated molecular and cellular events that proceed eventual tissular adaptations evidenced by the growth, stabilization, or regression of muscle capillaries. At the molecular level, among a plethora of angiogenic factors, skeletal muscle angio-adaptation is primarily mediated by the pro- and anti- angiogenic, vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (THBS1), respectively. At the cellular level skeletal muscle angio-adaptation involves endothelial cell proliferation, migration, and assembly into vascular tubes. Skeletal muscle angio-adaptation is then evidenced by tissular changes in muscle capillarization such as alterations in capillary density (CD) and capillary to fiber ratio (C/F).

1.4.1 Overview of the molecular aspects of skeletal muscle angio-adaptation.

At the molecular level, skeletal muscle angio-adaptation refers to an intricate balance between a plethora of pro- and anti- angiogenic molecules (Egginton and Birot, 2014; Hellsten

and Hoier, 2014; Lemieux and Birot, 2021; Olfert, 2016; Olfert et al., 2015; Olfert and Birot, 2011). Despite an abundance of angiogenic molecules, the use of transgenic models has identified two key regulators of skeletal muscle angio-adaption, the pro-angiogenic vascular endothelial growth factor-A (VEGF-A), and the anti-angiogenic thrombospondin-1 (THBS1) (Malek and Olfert, 2009; Olfert et al., 2010, 2009).

VEGF-A is mitogenic growth factor critical for stimulating endothelial cell proliferation, migration, and vascular permeability (Hoier and Hellsten, 2014). This secretable glycoprotein has several different isoforms (121, 165, 189, 206 amino-acids in human) arising from alternative mRNA splicing (Ferrara et al., 1991; Gavin, 2009; Hoier and Hellsten, 2014; Houck et al., 1991; Neufeld et al., 1999; Park et al., 1993). Of the several identified isoforms of VEGF-A, isoform 165 has been described as the most abundant (Hoier and Hellsten, 2014; Ishii et al., 2002; Neufeld et al., 1999; Ng et al., 2001; Wellmann et al., 2001). Myofiber-specific VEGF-A knock-out mouse models reveal the importance of myocyte-derived VEGF-A to skeletal muscle capillarization. Transgenic VEGF-A juvenile mice (myofiber -/-VEGF-A) show a reduction in skeletal muscle capillarization which in turn results in a 34%, and 81% decrease in maximal running speed, and endurance capacity, respectively (Olfert et al., 2009; Tang et al., 2004). Furthermore, exercise training, a well known angiogenic stimulus, fails to re-establish basal capillarization in these models (Olfert et al., 2010). More recently, transgenic VEGF-A models specifically targeting skeletal muscle VEGF-A (skmVEGF-A) expression have been implemented in adult mice in which capillary networks are extensively established (Delavar et al., 2014; Knapp et al., 2016). As opposed to previous studies performed in juvenile mice (Olfert et al., 2010, 2009), the deletion of VEGF-A in the skeletal muscle of adult mice has no effect on existing muscle capillaries or exercise performance (Delavar et al., 2014). Yet, a clear effect of a reduced angiogenic response is observed with -/-skmVEGF-A mice, as exercise training does not improve capillarization in the soleus, gastrocnemius, or extensor digitorum longus (Delavar et al., 2014). However, despite these observations, exercise training increased C/F ratio, and CD, by 18%, and 25%, respectively, in the plantaris of adult -/-skmVEGF-A mice (Delavar et al., 2014). This insinuates that despite the importance of pro-angiogenic factors such as the well described VEGF-A, other molecular actors may play a substantive role in skeletal muscle angio-adaptation, one of these molecules deserving interest is certainly THBS1.

Similarly, to VEGF-A, THBS1, is a key contributor to the angio-adaptive balance. Yet, in contrast to VEGF-A, THBS1 interaction with endothelial cell surface receptors attenuates proliferation and migration, and stimulates apoptosis (Lawler and Lawler, 2012). THBS1 regulation of angiogenesis can also extent beyond direct interaction with endothelial cells. THBS1 can directly antagonize VEGF-A bioactivity through inhibiting VEGF-A release from the extracellular matrix, by directly binding to VEGF-A, and by facilitating VEGF-A uptake and clearance from circulation (Greenaway et al., 2007; Gupta et al., 1999; Rodriguez-Manzaneque et al., 2001; Wang et al., 2004). With respect to skeletal muscle angio-adaptation, THBS1 is identified as a key regulator through transgenic models described by Malek and Olfert (Malek and Olfert, 2009). Global deletion of THBS1 results in greater capillarity in plantaris, soleus, and gastrocnemius muscles with functional improvements in maximal running speed, and time to exhaustion (Malek and Olfert, 2009). Furthermore, chronic delivery of ABT-510, a THBS1 mimetic, reduces skeletal muscle capillarity under physiological conditions (Audet et al., 2013). The impact of THBS1 on skeletal muscle angio-adaptation is further illustrated when describing muscle capillarization as a function of a VEGF-A/THBS1 ratio. Our lab (Roudier et al., 2010) has reported a 23% reduction in C/F ratio in the soleus of 9-day hindlimb unloaded rats. In these animals, no change was found in the expression of VEGF-A in the soleus, however a 185% increase in THBS1 protein expression was observed, resulting in a 73% reduction in soleus VEGF-A/THBS1 ratio. Furthermore, in type 1 diabetic rat models, C/F ratio is reduced by 21%, and 15%, in the soleus, and plantaris, with an accompanied reduction in the VEGF-A/THBS1 ratio by 71% and 52%, respectively (Aiken et al., 2019). Interestingly, these observations were not influenced by VEGF-A protein expression with no change, and a 30% increase reported in the soleus and plantaris, respectively. Finally, in human subjects with chronic obstructive pulmonary disorder, exercise training for 6-weeks resulted in a 16% and 29% increase in vastus lateralis C/F ratio, and CD, respectively (Gouzi et al., 2013). Despite this observation, there was no change in VEGF-A protein expression, however, a strong and significant decrease (-44%) was found in THBS1 protein expression, resulting in a 65% increase in the VEGF-A/THBS1 ratio. Taken together, these findings suggest that between VEGF-A and THBS1, THBS1 may in fact be more sensitive to external stressors and be the real key regulator of the angio-adaptive process. For instance, capillary regression has been suggested to correlate better with changes in THBS1 rather than VEGF-A (Olfert, 2016).

In the case of secreted angiogenic molecules, not only is the expression of the ligand protein relevant but also the activity of the respective receptor(s) (Figure 2). Endothelial cells can express various VEGF-A receptors, of these, there are three different VEGF-receptors: VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4) (Olsson et al., 2006). Of these VEGF-receptors, VEGFR2 has been suggested as the most important for angio-adaptative processes mediated by VEGF-A. Binding of VEGF-A to VEGFR2, results in the dimerization, and autophosphorylation of the receptor resulting in downstream cascades stimulating endothelial cell proliferation, migration, vascular permeability, and survival (Hoier and Hellsten, 2014; Wang et al., 2020). In particular, the phosphorylation of VEGFR2 at residue Y1175 is critical for downstream signalling related to endothelial cell angiogenic responses (Takahashi et al., 2001; Wang et al., 2020). However, VEGFR2 also represents an additional avenue of THBS1 regulation of angiogenesis. In physiological conditions, VEGFR2 is found in complex with THBS1 receptors, cluster of differentiation 36 (CD36) and cluster of differentiation 47 (CD47) (Chu et al., 2013; Kaur et al., 2010; Morandi et al., 2021; Zhang et al., 2009). Pre-treatment of endothelial cells with 3TSR (THBS1 derived CD36 binding peptide) has been shown to reduce VEGF-A mediated phosphorylation of VEGFR2 through a CD36 dependant mechanism (Chu et al., 2013; Zhang et al., 2009). By binding to CD36, THBS1 stimulates the recruitment of Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP1) to the VEGFR2/CD36 complex. Recruitment of SHP1 attenuates VEGF-A stimulated phosphorylation of VEGFR2 at residue Y1175, inhibiting endothelial cell migration and tube formation (Chu et al., 2013; Morandi et al., 2021). Furthermore, binding of THBS1 to CD36 can lead to development of CD36 nanoclusters and the recruitment of Proto-oncogene tyrosine-protein kinase Fyn (Fyn). Phosphorylation of Fyn can then lead to downstream signalling inducing endothelial cell apoptosis through caspase-8 and -9 mechanisms (Jiménez et al., 2000; Morandi et al., 2021). In comparison, by binding to CD47, THBS1 induces the dissociation of the CD47/VEGFR2 complex, in turn this dissociation inhibits VEGF-A-stimulated phosphorylation of VEGFR2 at residue Y1175 (Kaur et al., 2010; Morandi et al., 2021). Pre-treatment of endothelial cells with 7N3 (THBS1 derived CD47 binding peptide) results in reduced association of VEGFR2 with CD47 in the presence of VEGF-A, and reduces VEGF-A phosphorylation of VEGFR2 (Kaur et al., 2010). Between the two receptors (CD36 and CD47) there appears to be redundant functioning, however CD47 may be more relevant. For example, when pre-treating endothelial cells with binding peptides for CD36 (3TSR) or CD47 (E3CaG) at

physiological concentrations of THBS1 (2nM), a significant reduction of VEGFR2 activation in the presence of VEGF-A was only observed with E3CaG (Kaur et al., 2010). Thus, during physiological circumstances, CD47 may be responsible for THBS1 regulation of VEGFR2 activation rather than CD36.

FIGURE 2

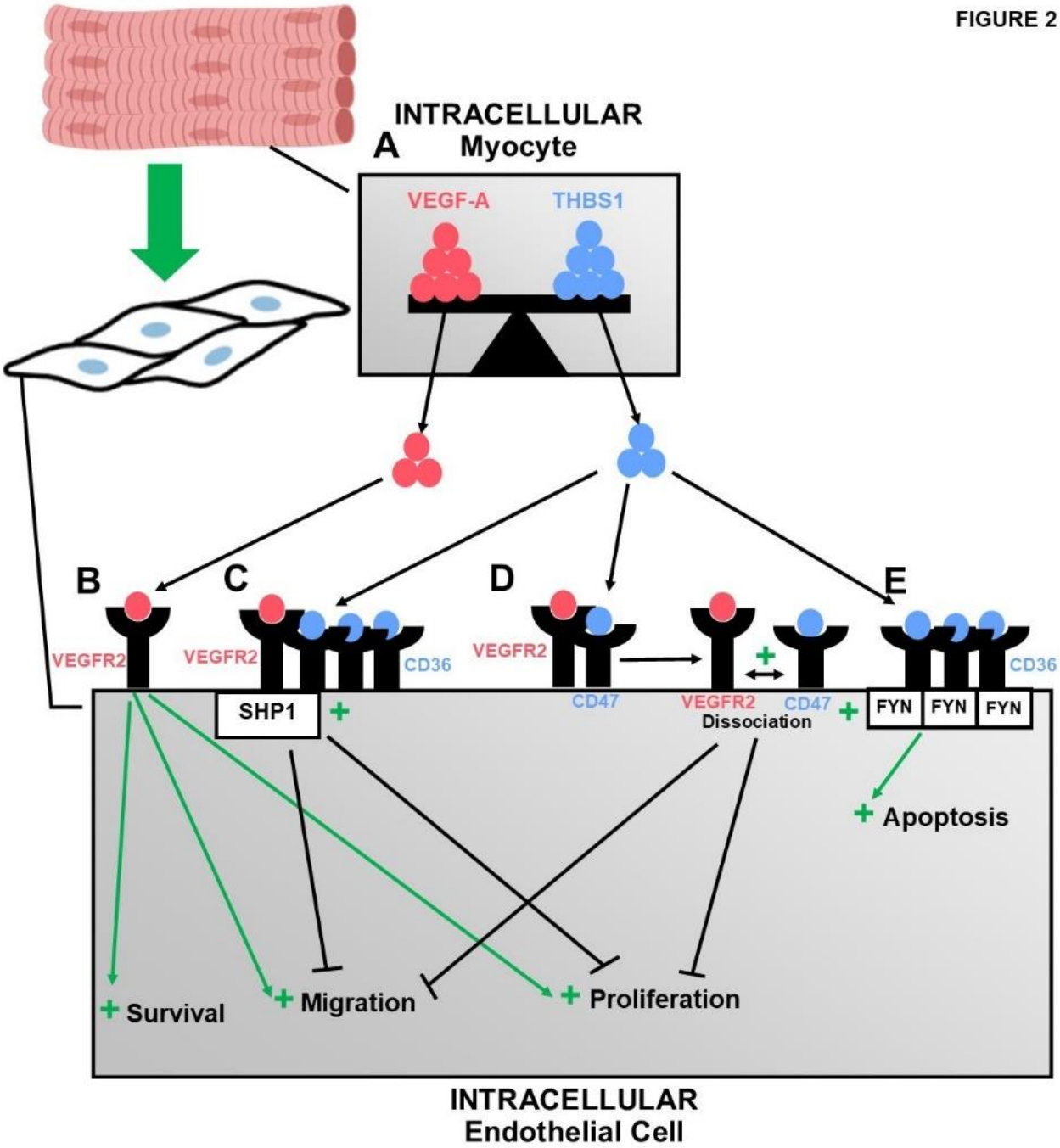

Figure 2. Schematic representation of molecular interactions between ligands VEGF-A and THBS1 to endothelial cell surface receptors VEGFR2, CD36 and CD47.

(A) Skeletal muscle cells are a source of production of both the pro- and anti- angiogenic vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (THBS1), respectively. **(B)** Interaction of VEGF-A to endothelial cell surface receptor vascular endothelial growth factor receptor -2 (VEGFR2) initiates pro-angiogenic downstream signalling cascades promoting endothelial cell proliferation, migration, and survival. **(C)** Binding of THBS1 to CD36 induces the recruitment of phosphatase SHP1 to the VEGFR2/CD36 complex resulting in reduced phosphorylation of VEGFR2 in the presence of VEGF-A and attenuation of pro-angiogenic downstream signalling. **(D)** In the absence of THBS1, VEGR2 and CD47 form a constitutive complex. Upon THBS1 ligation to CD47, there is a dissociation of the VEGFR2/CD47 complex leading to reduced VEGF-A phosphorylation of VEGFR2 and downstream signalling related to endothelial cell proliferation and migration. **(E)** Binding of THBS1 to CD36 leads to recruitment and phosphorylation of Fyn stimulating downstream cascades related to endothelial cell apoptosis.

As stated previously, skeletal muscle angio-adaptation is dependant on a plethora of pro- and anti- angiogenic factors. Yet, many of these factors respectively may function as either pro- or anti- angiogenic and can differ in expression based on physiological or pathological circumstances. For example, angiomotin is a transmembrane receptor that has two isoforms, p80 and p130. The p80 isoform is strongly involved in stimulating endothelial cell migration both *in-vitro* and *in-vivo*, whereas the p130 isoform weakly stimulates endothelial cell migration but is tightly associated to cytoskeleton actin filaments involved in vessel stabilization and migration. Our lab (Roudier et al., 2009) has shown exercise training to improve muscle CD (24% vs. 25%, respectively) and C/F (24% vs. 24%, respectively) to similar extents in both lean and obese animals. In both groups of animals, exercise training resulted in an improved p80/p130 ratio. However, in lean animals this was achieved by a 62% increase in the p80 isoform, whereas in obese animals the p80 isoform was unchanged but a 59% reduction in the p130 isoform was observed. Thus, while VEGF-A and THBS1 are key angiogenic molecules, stating that skeletal muscle angio-adaptation would completely depend on these two factors only would obviously be a reductionist perspective and fails to illustrate the molecular complexity.

1.4.2 Overview of the cellular aspects of skeletal muscle angio-adaptation.

At the cellular level, skeletal muscle angio-adaptation refers to the ability of skeletal muscle endothelial cells to proliferate, migrate, and assemble into vascular tubes in the context of angiogenesis. Overall, angiogenesis is a highly coordinated process. The main steps involved are: the activation of quiescent endothelial cells to form a vessel sprout; the elongation of the sprout involving endothelial cell proliferation and migration; and finally, the stabilization of a newly anastomose vessel into a mature blood vessel (Carmeliet and Jain, 2011). An exhaustive list of molecular factors facilitate these processes such as VEGFs, fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs), angiopoietins, integrins and proteases, to name a few (Carmeliet and Jain, 2011). Redundant functioning and overlapping signalling between many of these angiogenic factors, and within respective signalling cascades, highlights the complexity of angiogenesis. For instance, VEGFR2 phosphorylation can promote both endothelial cell proliferation and migration through mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) signalling (Muñoz-Chápuli et al., 2004). Furthermore, endothelial cells display a wide range of heterogeneity with differences in size, shape, position of nucleus, intracellular junctional contacts, and the expression of surface antigens (Auerbach et al., 1985; Belloni and Nicolson, 1988; Conway and Carmeliet, 2004). For example, when examining the gene expression profile of 52 different endothelial cell types from 14 different locations, Chi and colleagues (Chi et al., 2003) found differences in gene expression between large vessel and microvascular endothelial cells, and between microvascular endothelial cell populations based on tissue specificity. Therefore, with a plethora of molecular factors involved in angiogenic processes and a high degree of heterogeneity between endothelial cells used to delineate these molecular cascades, it is difficult to purvey a concise summary of endothelial cell responses during angiogenesis. However, with respect to skeletal muscle angio-adaptation, exercise provides an effect model to highlight specific endothelial cell angiogenic responses.

For example, Jensen and colleagues (Jensen et al., 2004) examined the effect of a knee-extensor training program on skeletal muscle capillarization and endothelial cell proliferation. Four weeks of exercise training resulted in a significant improvement in CD and C/F ratio in vastus lateralis biopsies of trained legs. Immunohistological staining for marker of proliferation ki-67 (Ki-67) and cluster of differentiation 31 (CD31) positive cells revealed a 1700% increase in the

number of proliferating cells associated with capillaries in these same biopsy samples. Furthermore, microdialysate collected post acute-exercise from probes inserted in vastus lateralis muscle, from both untrained and trained legs, stimulated a 573% and 415% increase in endothelial cell proliferation *in-vitro*, respectively. Complementary to these observations, our lab (Aiken et al., 2016) has used an elegant three dimensional muscle explant assay demonstrating the effect of an acute bout of exercise on endothelial cell migration. After 6 days of muscle incubation, plantaris biopsy samples from exercised rats demonstrated 118% greater endothelial cell migratory outgrowth compared to sedentary conditions. Together, these findings illustrate the sensitivity of endothelial cell proliferative and migratory responses to a transient angiogenic stimulus in the form of an acute bout of exercise. However, it should be noted that while endothelial cell proliferation and migration are crucial aspects of angiogenesis, they are by no means entirely reflective of endothelial cell behaviour involved in skeletal muscle angio-adaptation.

1.4.3 The concept of skeletal muscle angio-adaptation: Conclusion.

Skeletal muscle angio-adaptation refers to dynamic and coordinated processes that occur at molecular and cellular levels which influence the tissular plasticity of muscle capillaries (Lemieux and Birot, 2021; Olfert and Birot, 2011). Indeed, skeletal muscle angio-adaptation is well described in physiological and pathological conditions (Aiken et al., 2019; Andersen, 1975; Andersen and Henriksson, 1977; Gouzi et al., 2013; Roudier et al., 2013, 2012, 2010), however, things are less clear when it comes to the impact of environmental stressors.

1.5. Interest and relevance in studying and using environmental stressors in the context of skeletal muscle angio-adaptation.

Recently, there has been increasing interest in the use of environmental stressors as ergogenic aids to improving exercise performance or as therapeutic treatments in clinical settings. In particular, the use of ambient hypoxia superimposed with exercise training, or local muscle cooling in the form of cold-water immersion post-exercise, have gained popularity (Broatch et al., 2018; Girard et al., 2017; Ramos-Campo et al., 2019; Stephens et al., 2017). However, encounters with ambient hypoxia and cold stress are not limited to these instances. With increasing altitude, a reduction in barometric pressure leads to a reduced partial pressure of oxygen (PO_2), yet for every 1000m higher in elevation, temperature decreases by about 9.8℃ (West, 2012). Thus, high altitude represents a unique circumstance in which there is a combination of the stressors.

Interestingly, myofibers are sensitive to both ambient hypoxia and cold stress by having alterations in metabolic profile, contractile phenotype, and size (Broatch et al., 2018; Chaillou, 2018). Thus, the question arises, how are skeletal muscle capillaries influenced by these environmental stressors and, what is their effect on angio-adaptive processes? Answering these questions may not only be pertinent for those using ambient hypoxia and cold stress in sport-performance or clinical settings, but also for those that live or engage in activities at high altitude.

Currently, there is a discrepancy in original research conducted between the effect of ambient hypoxia and cold stress regarding skeletal muscle angio-adaptation. When conducting a *PubMed* search using the key words: Hypoxia, Skeletal muscle, Angiogenesis; 269 articles are rendered. In contrast, when searching: Cold, Skeletal muscle, Angiogenesis; 23 articles are rendered. Furthermore, when searching for the most well described angiogenic molecule: VEGF-A, Skeletal muscle, Hypoxia; 188 articles are found, in comparison, VEGF-A, Skeletal muscle, Cold; obtains 15 articles.

1.5.1. Investigating the impact of environmental stressors on skeletal muscle angio-adaptation.

Based on the discrepancy in previously conducted original research, my thesis will have two studies to address the influence of environmental stressors on angio-adaptation.

Study 1: A comprehensive literature review examining the respective and combined contributions of ambient/environmental hypoxia and exercise-induced local tissue hypoxia on skeletal muscle angio-adaptation.

Study 2: An original research project examining the effect of cold stress *in-vitro* on molecular and cellular aspects of skeletal muscle angio-adaptative processes. In particular, the impact of cold stress respectively on myotubes and skeletal muscle endothelial cells, and the influence of myotube derived angiogenic factors on endothelial cell responses.

2. STUDY 1

Altitude, Exercise, and Skeletal muscle Angio-adaptive Responses to Hypoxia: A Complex Story

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Author contributions

Both myself and my supervisor, Dr.Birot have equally contributed to formulating the design and content of the review, performing the literature search, and writing of the manuscript**.** I have personally designed and created Figure 4. Dr.Birot has personally designed and created Figure 3, Figure 5, and Figure 7. Both myself and Dr.Birot have equally contributed to the design and creation of Figure 6.

The use of this manuscript and the associated figures in this thesis has been approved by my supervisor and corresponding author on the manuscript, Dr.Birot (see Appendix A).

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The published form of this manuscript is attached to this thesis (see Appendix C).

2.1. Scientific context of the review.

Hypoxia, by definition, is a reduced availability of oxygen (Bhutta et al., 2022). Environmental hypoxia is also referred to as ambient hypoxia and is often associated with high altitude where a reduction in the partial pressure of oxygen (PO_2) occurs due to reduced barometric pressure (West, 2012). Aside environmental hypoxia, there are also physiological and pathological conditions in which the body can experience hypoxia at the tissue or cellular level independently of changes in barometric pressure. A non-exhaustive list of examples could be: intense aerobic exercise (Ameln et al., 2005; Lindholm and Rundqvist, 2016; Richardson et al., 1995), peripheral artery disease, chronic heart failure, and chronic obstructive pulmonary disorder (Chaillou, 2018; Semenza, 2014).

With respect to skeletal muscle, exercise imposes a greater tissular hypoxic stress than that of ambient hypoxia. Resting intramuscular PO_2 in muscles of the lower leg are reported to range between 13-34 mmHg at rest (Richardson, 2000; Richardson et al., 2006, 1999b, 1999a). When inspiring ambient air with a fraction of inspired oxygen (FiO_2) of 10% at rest (equivalent to an altitude of about 5,500m), intramuscular PO_2 is only reduced to 23 mmHg relative to 34mmHg in normoxia (Richardson et al., 2006). In comparison, when knee-extensor exercise is performed at 25% of work-rate max, intramuscular PO_2 is drastically reduced to 3.5 mmHg (Richardson et al., 2006). Interestingly, when subjects performed identical knee-extensor exercise whilst inspiring ambient air with a FiO2 of 10%, intramuscular PO_2 is further reduced to 2.3 mmHg (Richardson et al., 2006). Thus, when superimposing ambient hypoxia and physical exercise there is an exacerbation of the level of hypoxia experienced by the skeletal muscle.

With regard to skeletal muscle capillarization, previously established dogma suggests that ambient hypoxia stimulates an increased muscle capillary density (CD), however this would be a consequence of amyotrophy (reduced myofiber cross-sectional area) (Hoppeler, 1999; Hoppeler et al., 1990b; Hoppeler and Desplanches, 1992; Oelz et al., 1986). In contrast, exercise is a hypoxic stimulus that is well established in stimulating skeletal muscle angiogenesis (Ameln et al., 2005; Andersen, 1975, 1975; Lindholm and Rundqvist, 2016; Richardson et al., 1995). Interestingly, when the two stressors are combined, muscle capillarization improves to a larger extent than when performing exercise alone (Bigard et al., 1991; Desplanches et al., 1996, 1993; Olfert et al., 2001; Terrados et al., 1988; van Ekeren et al., 1992; Vogt et al., 2001). This response may be explained

by the fact that the expression of key angiogenic molecules (VEGF-A, THBS1) are influenced by both exercise, and ambient hypoxia, independently and when combined in skeletal muscle (Breen et al., 2008, 1996; Olfert et al., 2006; Yadav et al., 2014).

Therefore, the purpose of **Study 1** was to review the existing literature to provide the reader with an updated view on if/how ambient hypoxia, with or without an exercise stimulus, affects skeletal muscle angio-adaptation. Furthermore, **Study 1** was also completed to maintain a high-degree of productivity and actively contribute to the scientific community despite lockdown restrictions imposed during the COVID-19 pandemic.

2.2. Research question.

How does ambient hypoxia alone or when superimposed with exercise affect skeletal muscle angio-adaptation and tissular outcomes in muscle capillarization?

2.3. Hypothesis.

(1) Ambient hypoxia per se is not a sufficient stimulus to trigger true muscle angiogenesis.

Chronic exposure to ambient hypoxia results in an improved muscle CD. However, whether this occurs exclusively due to myofiber atrophy or due to a true angiogenic response (improved C/F) triggered by ambient hypoxia is debated. Furthermore, confounding variables found in field and laboratory experiments have made interpretations of the effect of ambient hypoxia *per se* difficult. Such confounding variables are the nutritional status of the animals or subjects, physical activity levels, the intensity and duration of exposure to hypoxia, the use of hypobaric versus normobaric hypoxia, the absence of proper control groups, and the influence of other environmental stressors in particular cold stress.

(2) Exercise training when performed under ambient hypoxia elicits an improved angiogenic response of the skeletal muscle compared to exercise training alone.

VEGF-A and THBS1 have been shown to be key molecules involved in skeletal muscle angio-adaptation through transgenic models (Delavar et al., 2014; Malek and Olfert, 2009; Olfert et al., 2010, 2009). Exercise performed under hypoxic conditions leads to a greater and longer persisting increase in VEGF-A expression in comparison to exercise alone (Breen et al., 1996). Furthermore, exposure to chronic ambient hypoxia reduces both basal and exercise-induced

expression of THBS1 (Olfert et al., 2006). Together, these results suggest that exercise under ambient hypoxia may favourably shift the VEGF-A/THBS1 balance towards a greater pro-angiogenic response than exercise alone.

2.4. Study aim.

1) Synthesize the existing literature in a comprehensive review that provides the reader with an updated view of the field.

2) Identify controversies and gaps of knowledge in the field that challenge the established dogma concerning the effect of ambient hypoxia on skeletal muscle capillarization.

3) Identify future research directions to advance this unique field of exercise and environmental physiology.

2.5. Methodology.

To investigate the effect of ambient hypoxia with or without exercise on influencing skeletal muscle angio-adaptation, we searched *PubMed* for all studies in which animal or human subjects were exposed to normobaric or hypobaric hypoxia, both with or without exercise (acute and exercise training), that had outcome measurements of muscle capillarization and/or angiogenic molecule expression.

2.6. Discussion.

Following the completion of **Study 1**, several key points have been identified.

First, when analyzing 22 articles that assessed skeletal muscle CD, and/or C/F ratio, in human subjects, and animal passively exposed to normobaric or hypobaric hypoxia, we found that with respect to CD, 55% of studies reported a 10% increase or greater, whereas 45% of studies showed no change. Based on these results we can challenge the conventional dogma that passive exposure to ambient hypoxia improves skeletal muscle CD. Furthermore, when investigating C/F ratio, a quantitative index of novel capillary growth, in the same 22 articles, we observe 13% of studies reporting an increase of 10% or more, 23% reporting a decrease of 10% or more, and 64% of studies reporting no change. Thus, this lack of consensus suggests that passive ambient hypoxia exposure might not stimulate angiogenesis, e.g., a true formation of new capillaries.

Second, combing exercise and hypoxia results in an improved angiogenic response than exercise alone.

Third, several studies have investigated the impact of passive hypoxia on skeletal muscle angio-adaptation by characterizing the expression of VEGF-A, yet, when analyzing these studies, we have identified flaws in the current literature. For one, many of these studies assessed VEGF-A expression at the mRNA level and when VEGF-A protein was measured, it was usually circulating VEGF-A being quantified by enzyme-linked immunosorbent assay (ELISA). Furthermore, many of these studies suggest changes in VEGF-A expression to be synonymous with a comprehensive angio-adaptive response. First, assessing VEGF-A mRNA does not necessarily reflect an increased biological activity of the molecule given that increased mRNA does not necessarily reflect greater translation of the protein. Second, measuring circulating VEGF-A by ELISA does not necessarily reflect skeletal muscle VEGF-A production. Finally, as mentioned previously skeletal muscle angio-adaptation is regulated by a plethora of pro- and anti-angiogenic molecules that promote endothelial cell responses towards muscle capillary remodeling. Thus, in these studies, measuring VEGF-A expression only does not fully encapsulate the dynamic and multi-stepped processes involved in skeletal muscle angio-adaptation. Therefore, we caution against the use of these studies as evidence for a, or lack there of, skeletal muscle angio-adaptive response to hypoxia.

Fourth, as mentioned in my literature review, THBS1 could impact skeletal muscle capillarization to a larger extent than VEGF-A, however, despite this THBS1 is remarkably understudied. Only a single study (Olfert et al., 2006) has investigated the combined impact of exercise and hypoxia on skeletal muscle THBS1 expression.

In conclusion, we found that our study comprehensively and effectively reviewed the literature, in doing so, we challenged existing dogma, identified gaps in knowledge, and provided directions for future research in environmental and exercise physiology. We also found that this review was well received in the scientific community with 4,052 views, 703 downloads, and 2 citations since its publication on Sept 6, 2021.

2.7. Original version of the published review.

Altitude, exercise, and skeletal muscle angio-adaptive responses to hypoxia: A complex story.

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Key words: Skeletal muscle; capillary; angiogenesis; hypoxia; altitude; exercise; VEGF-A; thrombospondin.

Abstract

Hypoxia, defined as a reduced oxygen availability, can be observed in many tissues in response to various physiological and pathological conditions. As a hallmark of the altitude environment, ambient hypoxia results from a drop in the oxygen pressure in the atmosphere with elevation. A hypoxic stress can also occur at the cellular level when the oxygen supply through the local microcirculation cannot match the cells' metabolic needs. This has been suggested in contracting skeletal myofibers during physical exercise. Regardless of its origin, ambient or exercise-induced, muscle hypoxia triggers complex angio-adaptive responses in the skeletal muscle tissue. These can result in the expression of a plethora of angio-adaptive molecules, ultimately leading to the growth, stabilization, or regression of muscle capillaries. This remarkable plasticity of the capillary network is referred to as angio-adaptation. It can alter the capillary-to-myofiber interface, which represent an important determinant of skeletal muscle function. These angio-adaptive molecules can also be released in the circulation as myokines to act on distant tissues. This review addresses

the respective and combined potency of ambient hypoxia and exercise to generate a cellular hypoxic stress in skeletal muscle. The major skeletal muscle angio-adaptive responses to hypoxia so far described in this context will be discussed, including existing controversies in the field. Finally, this review will highlight the molecular complexity of the skeletal muscle angio-adaptive response to hypoxia and identify current gaps of knowledges in this field of exercise and environmental physiology.

Introduction.

Our review aims to revisit the complexity of the skeletal muscle angio-adaptive response to hypoxia, particularly when combining exposure to ambient hypoxia and exercise-induced tissue hypoxia. Indeed, in the context of high-altitude expeditions, mountaineers usually engage into prolonged periods of intense physical activity over several weeks or months (West, 2012, 2006). In the context of sport performance at sea level, hypoxia training has become a complex and very specialized area of research (Girard et al., 2013; Girard and Chalabi, 2013; Girard and Pluim, 2013; Lundby et al., 2012; Millet et al., 2010). Finally, the use of exercise training under hypoxia has recently emerged as a new and promising therapeutic avenue to improve some metabolic and cardiovascular conditions (obesity, type-2 diabetes, hypertension) as well as for the training of elderly subjects (Millet et al., 2016; Verges et al., 2015) (Figure 3).

Skeletal muscles represent one of our largest tissues, accounting for about 40% of human body weight. Skeletal muscles adapt to environmental, physiological, and pathological conditions with a remarkable plasticity. This can include changes in muscle mass, in the size of myofibers and their metabolic and contractile phenotype, as well as changes in muscle capillarization (Booth and Thomason, 1991; Hudlicka, 2011; Hudlicka et al., 1992).

Since August Krogh's pioneering work about a century ago (Krogh, 1919a, 1919b, 1919c), our understanding of the regulation of muscle blood flow and oxygen delivery to muscle cells has considerably evolved and was recently revisited in great review articles (Angleys and Østergaard, 2020; Kissane et al., 2021; Poole et al., 2021, 2020). The oxygen cascade from skeletal muscle arterioles to capillaries, interstitial tissue, sarcolemma, and mitochondria can be influenced at several levels: The vasomotricity of upstream arterioles and the subsequent regulation of capillary blood flow; the content and velocity of red blood cells; the hemoglobin and myoglobin concentrations; the tortuosity and number of capillaries; and the surface area of myofibers.

The capillary-to-myofiber interface plays a crucial role for muscle function. Indeed, it represents the site of exchange for oxygen, nutrients, metabolic heat and waste between the blood and the myofibers. The density of capillaries within a given area of muscle tissue will greatly contribute to matching the delivery of oxygen and nutrients with the myofibers' metabolic needs, particularly during contractile activity (Hoppeler, and Kayar, 1988; Hudlicka, 2011; Hudlicka et al., 1987; Mathieu-Costello, 1994). The capillary network can therefore be considered as a key determinant of skeletal muscle function and several studies have reported strong correlations between the level of muscle capillarization and mitochondria volume density, muscle oxidative capacity, and oxygen consumption (Hoppeler et al., 1987; Hoppeler, and Kayar, 1988; Howlett et al., 2003; Hudlicka et al., 1987; Poole and Mathieu-Costello, 1996). For instance, Howlett et al. (2003) reported a strong correlation between skeletal muscle capillary density and muscle oxygen conductance in rats selectively bred for running endurance (Howlett et al., 2003).

Skeletal muscle capillarization and the concept of muscle angio-adaptation.

The capillary density in a muscle section can vary in response to various environmental, physiological, or pathological conditions. An increase in muscle capillary density is usually observed in human subjects and animal models in response to prolonged endurance training or high-altitude sojourn (Breen et al., 2008; Hudlicka, 2011; Hudlicka et al., 1992). Conversely, skeletal muscle capillary rarefaction has been described in response to physical deconditioning as well as in the context of some pathologies such as chronic obstructive pulmonary disease, chronic heart failure, or diabetes (Aiken et al., 2019; Gouzi et al., 2013; Olfert et al., 2015; Roudier et al., 2010).

Skeletal muscle angio-adaptation refers to the complex and dynamic processes of capillary formation, stabilization, or regression in response to acute and chronic physiological or pathological conditions. These processes are regulated at the molecular level by a plethora of pro- and anti- angiogenic molecules (Breen et al., 2008; Egginton, 2009; Egginton and Birot, 2014; Hoppeler, 1999; Olfert et al., 2015; Olfert and Birot, 2011). At the cellular level, endothelial cells proliferate, migrate, and assemble to form new capillaries in the context of angiogenesis, or conversely, undergo apoptosis during capillary regression. Myofibers can represent an important source of production and release of angio-regulatory molecules such as the well-described pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A).

Importantly, changes in capillary density can also be a direct consequence of alterations in the size of myofibers (hypertrophy or atrophy) without any true capillary loss or formation. Also, no change in capillary density is not necessarily synonymous of an absence of angio-adaptive activity. For example, during myofiber hypertrophy, a formation of new capillaries might occur simply to prevent a decrease in the capillary density that would result from the increase in the myofibers surface area. It is also important to note that some angio-adaptive molecules, such as the pro-angiogenic VEGF-A, are not only required for the growth of capillaries but also to maintain existing ones. Evidence suggests an autocrine expression of VEGF-A being important for endothelial cell survival and vascular homeostasis (Domigan et al., 2015; Lee et al., 2007). Finally, the skeletal muscle could also be seen as an endocrine organ releasing "angio-adaptive myokines" in circulation, potentially affecting distant tissues.

Skeletal muscle hypoxia: Impact of altitude and exercise.

Hypoxia, defined as a lack of oxygen supply to a given tissue, is a powerful pro-angiogenic stimulus for various cell types and tissues including skeletal muscle (Breen et al., 2008; Favier et al., 2015; Fraisl et al., 2009; Hoppeler and Vogt, 2001; Semenza, 2001). Ambient hypoxia is a hallmark of the altitude environment and results from a drop in the oxygen pressure in the atmosphere with elevation. However, the impact of ambient hypoxia *per se* on skeletal muscle angio-adaptive responses and capillarization still remains a source of scientific debate. A hypoxic stress can also be generated locally at the muscle tissue level in response to intense exercise if oxygen delivery cannot match the metabolic needs of contracting myofibers (Figure 3).

Several studies have aimed at determining the oxygen partial pressure (PO_2) cascade in human and animal models using different techniques such as proton magnetic resonance spectroscopy, surface electrodes, microcatheter, and more recently phosphorescence quenching. The intramuscular PO_2 at rest is estimated around 27 mmHg with some variations (10-34 mmHg) among studies (different species, muscles and techniques), below the microvascular PO_2 but well above the intra-myocyte PO_2 (1-3 mmHg) (Boegehold and Johnson, 1988; Colburn et al., 2020; Hirai et al., 2019, 2018; Hutter et al., 1999; Johnson et al., 2005; Kindig et al., 2003; Liu et al., 2012; Lombard et al., 2000; Molé et al., 1999; Poole et al., 2021, 2020; Prewitt and Johnson, 1976; Richardson, 2000, p. 200; Richardson et al., 2006, 1995; Richmond et al., 1999, 1997).

A few of these studies have investigated the impact of ambient hypoxia on skeletal muscle PO_2. A modest reduction in PO_2 (from 34 mmHg under normoxia to 23 mmHg under hypoxia) was observed in human quadriceps muscle following an exposure to an inspired O_2 fraction of 10% (F_iO_2 0.10), equivalent to an altitude of about 5,800 m. (Richardson et al., 2006). In another study, the interstitial PO_2 in rat cremaster muscles was reduced from 26 to about 10 mmHg following one minute exposure to an inspired O_2 fraction of 7% (F_iO_2 0.07), equivalent to an altitude of about 8,300 m. (Johnson et al., 2005). However, the physiological relevance of this conditioning (1 minute of exposure at F_iO_2 0.07) can be questioned. These few studies suggest that ambient hypoxia exposure might not alter muscle PO_2 to a large extent. Conversely, the impact of physical exercise on the muscle PO_2 seems much more important. One bout of exercise, even at moderate intensity (50% maximal leg O_2 uptake) was shown to decrease muscle PO_2 to values around 3-5 mmHg (Angleys and Østergaard, 2020; Molé et al., 1999; Richardson et al., 2006, 1995). This exercise-induced hypoxic stress could be further exacerbated (muscle PO_2 down to 2 mmHg) when exercise was performed in a hypoxic environment (Richardson et al., 2006).

At a molecular level, the Hypoxia Inducible Factor-1 (HIF-1) is a transcription factor widely recognized as a hallmark of the hypoxia signaling pathway (Semenza, 2001; Semenza and Wang, 1992). HIF-1 is a heterodimeric complex formed of two subunits alpha and beta. Whereas the expression of the beta subunit remains stable under both normoxic and hypoxic conditions, the alpha subunit (HIF-1α) confers on HIF-1 most of its regulation. Under normoxia, the HIF-1α protein is constantly synthesized in the cytoplasm, and rapidly undergoes proteosomal degradation within a few minutes. This involves HIF-1α hydroxylation on certain proline residues by HIF prolyl hydroxylases (PHD1-3) (Ke and Costa, 2006; Lindholm et al., 2014). If the PO_2 drops enough to generate a hypoxic stress at the cellular level, the function of PHD1-3 is inhibited, HIF-1α is stabilized and can translocate into the nucleus to dimerize with the beta subunit. HIF-1 binds to hypoxia responsive elements (HRE) in the promoter regions of target genes to regulate their transcription. Both the erythropoietin (EPO) and pro-angiogenic VEGF-A genes possess HRE sites in their promoters (Semenza, 2001).

Whether ambient hypoxia exposure results in an increased expression of HIF-1α protein in skeletal muscle remains largely understudied. Stroka et al. (2001) have observed a strong HIF-1α protein expression level in mouse skeletal muscle even under normoxic conditions (Stroka et al., 2001). In the same study, one hour exposure to extreme normobaric hypoxia (F_iO_2 0.06, equivalent to

9,100 m) did not seem to increase the expression level much further. As noted previously, the physiological relevance of conditionings combining very short exposures (1 min. to 1 hour) and extreme hypoxia levels (F_iO_2 0.06-0.07) are questionable (Johnson et al., 2005; Stroka et al., 2001). Intense physical exercise can be at the origin of a local hypoxic stress in the muscle tissue. In an elegant study, Ameln et al. (2005) quantified HIF-1α protein expression in response to one single bout of moderate intensity exercise in human vastus lateralis muscle biopsies (Ameln et al., 2005). HIF-1α protein levels were increased immediately after exercise and remained elevated for up to 6h post-exercise. An increased nuclear staining for HIF-1α was also observed as well as an increased DNA binding to HRE binding sites. mRNA levels for HIF-1 target genes EPO and VEGF-A were also higher post-exercise. These results suggest that HIF-1 expression and activity were both increased post-exercise in human skeletal muscle.

An increase in HIF-1α protein expression could result from the inhibition of HIF-1α protein degradation but also from an increased expression of HIF-1α mRNA and protein translation. Vogt et al. (2001) have measured HIF-1α mRNA in human vastus lateralis muscle biopsies before and after an endurance training program conducted at low or high intensity either under normoxia or normobaric hypoxia (simulated altitude of 3,850 m) (Vogt et al., 2001). HIF-1α mRNA expression was increased after hypoxia training regardless of the training intensity whereas training under normoxia had no effect. Interestingly, VEGF-A mRNA levels were also measured and found to be increased only in the high-intensity hypoxia training group but not under low exercise training conditions. This could reveal a synergetic effect of combining ambient hypoxia and exercise-induced local hypoxia. Here, biopsies were performed at least 24h post-exercise to assess the effect of prolonged training on HIF-1α basal levels. In line with this study, Lundby et al. (2006) have evaluated the impact of prolonged exercise training on HIF-1α acute response to one single bout of exercise (Lundby et al., 2006). HIF-1α mRNA levels were increased in vastus lateralis biopsies at 6 hours post-exercise only in the untrained group. Whether exercise training could blunt the acute HIF-1α response to one single bout of exercise was then questioned by Lindholm and colleagues who showed that several inhibitors of HIF-1α expression and HIF-1 activity were increased in trained muscles (Lindholm et al., 2014; Lindholm and Rundqvist, 2016). The analysis of trained muscle biopsies indeed revealed higher expression levels of prolyl hydroxylases (PHD1-3), Factor Inhibiting HIF-1 (FIH) and Sirtuin-6 (SIRT6) (Lindholm et al., 2014). By catalyzing

hydroxylation on specific prolyl residues, PHD1-3 target HIF-1α for proteasomal degradation. FIH, a HIF-1α-specific asparagine hydroxylase, can inhibit HIF-1 transactivation. SIRT6, a histone deacetylase, can act as an epigenetic co-repressor of HIF-1. Another explanation to the results from Lundby et al. could be an increased level of muscle capillarization post-training (Lundby et al., 2006). Angiogenesis is a well-described tissular adaptation of the skeletal muscle tissue to endurance training. This would result in a better capillary-to-myofiber interface and oxygen delivery to contracting myofibers, and as such, a reduced exercise-induced hypoxic stress in trained muscles.

In addition to inducing a local cellular hypoxic stress, exercise combines other pro-angiogenic stimuli such as increased shear stress on endothelial cells due to enhanced muscle blood flow, mechanical tissue stretch, and oxidative stress (Breen et al., 2008; Egginton, 2009; Egginton and Birot, 2014; Haas and Nwadozi, 2015; Hellsten and Hoier, 2014; Hoier et al., 2012; Hudlicka et al., 1992; Milkiewicz et al., 2001; Olfert et al., 2015). The increase in muscle blood flow results from active vasodilation, and the resultant increase in shear stress represents a well-described pro-angiogenic stimulus for skeletal muscle endothelial cells, mediating the production of angio-adaptive molecules such as nitric oxide, metalloproteinases, and VEGF-A (Egginton, 2011, 2009; Haas and Nwadozi, 2015; Hellsten and Hoier, 2014; Hudlicka and Brown, 2009; Milkiewicz et al., 2011, 2011, 2001). If local cellular hypoxia and shear stress are often presented side by side as exercise-induced pro-angiogenic stimuli, passive exposure to ambient hypoxia also stimulates vasodilation and increases blood flow in skeletal muscle (Casey and Joyner, 2012, 2011; Dinenno, 2016; Joyner and Casey, 2014). Therefore, ambient hypoxia could then represent an upstream stimulus of muscle shear stress. The degree of vasodilation observed seems linked to the degree of ambient hypoxia, at least for acute exposures. Interestingly, the combination of ambient hypoxia exposure and exercise seems to synergistically induce greater vasodilation and muscle blood flow than what could be expected by simply adding their respective contributions (Casey and Joyner, 2012, 2011; Joyner and Casey, 2014). Finally, to add more complexity, HIF-1α expression can also be stimulated under normoxic conditions in skeletal muscle in response to increased shear stress or mechanical tissue stretch (Milkiewicz et al., 2007).

Altitude, exercise, and skeletal muscle hypoxia: Key points.

- Exercise is a more powerful stimulus than ambient hypoxia to decrease muscle PO_2.
- Combining ambient hypoxia and exercise might further decrease muscle PO_2.
- HIF-1 is a well-established hallmark of hypoxia signaling.
- Skeletal muscle angio-adaptive activity can locally change the level of muscle capillarization and can also release angio-adaptive myokines in the circulation for distant effects.

Increased muscle capillarization in response to exercise training and ambient hypoxia.

We previously discussed how increasing the capillary-to-myofiber interface could be beneficial for maintaining or improving muscle function when oxygen delivery becomes a challenge. The capillary density (CD), which represents the number of capillaries per surface unit of tissue, is often used to assess the level of muscle capillarization (Andersen, 1975; Hudlicka et al., 1992). The CD can however be influenced both by changes in the number of capillaries and alterations in the size of myofibers. Changes in CD might therefore not always be representative of angiogenesis or capillary regression. Conversely, the capillary-to-fiber ratio (C/F) represents one of the best histological parameters to truly appreciate capillaries formation or rarefaction.

Exercise training is a powerful and well-established pro-angiogenic stimulus for the skeletal muscle tissue (Andersen, 1975; Andersen and Henriksson, 1977; Egginton, 2009; Hudlicka et al., 1992). Human and animal studies have shown that one single bout of exercise leads to the production and release of several angio-adaptive molecules both in the muscle microenvironment, for example to stimulate skeletal muscle endothelial cells (Aiken et al., 2016; Roudier et al., 2012), and in the circulation as myokines (Egginton, 2009; Egginton and Birot, 2014; Hoier et al., 2012; Hudlicka et al., 1992; Olfert and Birot, 2011; Vogt et al., 2001). During prolonged exercise training, the chronic repetition of these exercise-induced angiogenic responses can ultimately lead to the formation of new capillaries. This increase in muscle capillarization will usually be reflected by higher C/F and CD in trained muscles (Andersen, 1975; Andersen and Henriksson, 1977; Egginton, 2009; Hudlicka et al., 1992). As previously mentioned, the exercise stimulus combines several stressors such as local tissue hypoxia, increased shear stress, tissue stretch, and oxidative stress. The exact contribution of hypoxia *per se* during exercise remains difficult to study and still unclear. Conversely to regular exercise, muscle hypokinesia or deconditioning can lead to capillary

regression (Egginton, 2009; Fujino et al., 2005; Malek et al., 2010; Olfert and Birot, 2011; Roudier et al., 2010).

The effect of passive exposure to ambient hypoxia on skeletal muscle capillarization is less clear than the impact of exercise training. A well-established consensus of the literature is that prolonged exposure to field or simulated high altitude hypoxia results in improved skeletal muscle capillarization and increased capillary density. This alteration in capillary density seems however mainly due to the atrophy of myofibers rather than a true angiogenic response. Several studies have indeed reported an increase in CD concomitantly to a decrease in the myofiber surface area, with no change in the C/F ratio (Green et al., 1989; Hoppeler et al., 1990b; Kayser et al., 1996; MacDOUGALL et al., 1991; Oelz et al., 1986).

This should be taken with a certain caution since there are in fact almost as many original studies reporting an increase in muscle CD as studies showing no change (Figure 4A) (Banchero et al., 1976; Bigard et al., 1991; Green et al., 1992; Hoppeler et al., 1990b; Kayser et al., 1996; Levett et al., 2012; Lundby, 2004; Mizuno et al., 2008; Oelz et al., 1986; Olfert et al., 2001; Poole and Mathieu-Costello, 1989; Sillau and Banchero, 1977). We searched the PubMed database for original research studies that analyzed skeletal muscle CD and C/F in animals or human subjects passively exposed to normobaric or hypobaric hypoxia. Studies combining ambient hypoxia and exercise interventions were included only if they had all the required experimental groups to assess the impact of passive hypoxia exposure independently of the exercise training stimulus. We identified a total of 22 original research studies (Figure 4). Interestingly, 55% reported a significant increase or a trend for an increase in CD by at least 10% whereas 45% showed no change (Figure 4A).

The lack of change in CD could be attributed to an absence of muscle atrophy, and some authors have in fact observed no reduction in myofiber size at high altitude (D'Hulst et al., 2016; Green et al., 1992; Levett et al., 2012; Lundby, 2004). It was then suggested that both the exposure duration and the altitude level could determine whether myofibers would atrophy or not, leading to the notion of hypoxic dose (D'Hulst and Deldicque, 2017; Millet et al., 2017). Some conditionings were indeed performed at moderate altitude (3000-4000 m) over 2-3 weeks only, as opposed to longer exposures (8-10 weeks) at higher altitudes (>5200 m) (Green et al., 1992, 1989; Hoppeler et al., 1990b; Levett et al., 2012; Lundby, 2004; MacDOUGALL et al., 1991; Mizuno et al., 2008; Oelz et al., 1986).

It is difficult to identify the source of discrepancy between these studies regarding myofiber size and CD. In rodent studies for example, the age of the animals can be a confounding factor if they are still growing (Banchero, 1985; Snyder et al., 1992). Calorie intake can also influence body and muscle weights. Prolonged mountaineering expeditions at high-altitude can involve logistical constraints for proper nutrition and gastroenteritis disorders are often described (Swenson and Bärtsch, 2013; West, 2012). Hypophagia has been well observed in rodents exposed to prolonged hypoxia, usually requiring the use of pair-fed control animals (Daneshrad et al., 2003, 2001).

Muscle atrophy could also result from reduced physical activity and muscle deconditioning, particularly during prolonged and passive exposure in hypoxic chambers. For example, when maintaining an isocaloric state and matching physical activity levels, Green and colleagues did not observe any atrophy in their study (Green et al., 1992). There is however still a lack of consensus around the contribution of physical activity or the notion of a hypoxic dose on muscle atrophy. This can nicely be illustrated by two studies from Mizuno and Levett (Levett et al., 2012; Mizuno et al., 2008). Both compared very active versus less active subjects during similar conditions of prolonged exposure (66-75 days) to high altitude (>5000 m). Whereas the less active participants remained moderately active at the Everest Base Camp (5250-5300 m), active subjects were repeating climbing sessions at higher altitudes. In Levett's study, climbers reached Camp 2 (6400 m) and some even summited the Mount Everest (8848 m) (Levett et al., 2012). Mizuno et al. (2008) observed a significant increase in muscle CD (+14%) associated with a reduction in muscle circumferences and myofiber size (Mizuno et al., 2008). Conversely, Levett et al. (2012) did not observe any significant myofiber atrophy or change in CD (Levett et al., 2012).

Some authors have also concluded that ambient hypoxia alone might not be sufficient to stimulate skeletal muscle angiogenesis and that a combination of cold and hypoxia stressors might be required (Banchero, 1985; Hoppeler, 1999; Jackson et al., 1987; Snyder et al., 1992). Interestingly, cold *per se* was recently identified as a pro-angiogenic stimulus in rodent skeletal muscle (Deveci and Egginton, 2003; Egginton, 2002; Sillau et al., 1980).

An increase in muscle CD can also result from the formation of new capillaries via the process of angiogenesis. The capillary-to-fiber ratio (C/F) is often used to assess capillary formation. Some earlier studies in species adapted to high altitude, including deer mice, gooses, finches or pigeons have reported increased C/F values compared to sea level animals (Hepple, 2000; Hepple et al., 1998; Lui et al., 2015; Mathieu-Costello and Agey, 1997; Scott et al., 2015). Yet, as previously

mentioned, a general interpretation is that the increase in CD in response prolonged exposure to hypoxia results from tissue remodeling and myofiber atrophy without any angiogenesis. Methodological artefacts in tissue preparation for histology procedures, such as variation in the sarcomere length, were pointed out (Banchero, 1985; Banchero et al., 1985; Hoppeler et al., 1990b; Hoppeler, and Kayar, 1988; Hudlicka et al., 1992; Mathieu-Costello, 1994; Poole and Mathieu-Costello, 1989, 1989; Sillau et al., 1980; Sillau and Banchero, 1977). The impact of prolonged hypoxia on muscle C/F was however revisited by Deveci and colleagues in rats passively exposed to 3 or 6 weeks of hypoxia (F_iO_2 0.12, equivalent to 4400 m) (Deveci et al., 2002, 2001). The effect of hypoxia on C/F could vary accordingly to muscle and fiber types as well as exposure duration. After 3 weeks of conditioning, CD and C/F were increased in the diaphragm and soleus muscles without any significant myofiber atrophy, suggesting a true angiogenic response to ambient hypoxia. No indication of angiogenesis was observed in the tibialis anterior and extensor digitorum longus muscles (Deveci et al., 2001). At 6 weeks, an increase in C/F was observed all muscles (Deveci et al., 2002). Interestingly, the analysis of different areas from each muscle also suggested that angiogenesis might occurs predominantly around larger and glycolytic myofibers (Deveci et al., 2002, 2001).

When re-analyzing our 22 original research studies that measured the C/F ratio in animal and human skeletal muscles in response to prolonged exposure to hypoxia, we found that about 64% mentioned no change and only 13% reported a significant increase or a trend for it (Figure 4B). Interestingly, 23% indicated a significant or a trend for a decrease in C/F (Green et al., 1989; Hoppeler et al., 1990b, 1990a; Lundby, 2004; MacDOUGALL et al., 1991). For example, in the study from Hoppeler and colleagues, the significant decrease in C/F (-10%) is of the same magnitude as the increase in CD (+11%) (Hoppeler et al., 1990b).

Based on the current literature, the understanding of the effect of prolonged exposure to hypoxia on skeletal muscle capillarization is still unclear and difficult to generalize. Responses can vary between species, muscles, and fiber types, and can be different when combining different stressors such as cold or physical activity. The idea of CD increasing because of myofiber atrophy can be seen as an interesting and economic way to improve the capillary-to-myofiber interface without the need of angiogenesis. However, having smaller myofibers might represent a disadvantage when it comes to muscle performance.

Divergent from passive exposure to ambient hypoxia, the utilization of hypoxia in conjunction with exercise has gained popularity as a possible training avenue for improving sea-level exercise performance (Brocherie et al., 2018; Chapman, 2013; Girard et al., 2020, 2013; Girard and Chalabi, 2013; Levine, 2002; Levine and Stray-Gundersen, 2006; Lundby et al., 2012; Millet et al., 2010). As discussed earlier, exercise can generate a local hypoxic stress in the skeletal muscle tissue. It is therefore appealing to consider how the combination of ambient hypoxia and exercise training would affect muscle capillarization.

Several rodent and human studies suggest that skeletal muscle capillarization might improve to a larger extent when exercise training is performed under ambient hypoxia compared to sea level conditions (Bigard et al., 1991; Desplanches et al., 1996, 1993; Olfert et al., 2001; Terrados et al., 1988; van Ekeren et al., 1992; Vogt et al., 2001). To assess the impact of ambient hypoxia on the angiogenic effect of training, some authors trained their animals or subjects at the same relative intensity, for example at a similar percentage of VO_2max determined under hypoxia and normoxia, thus expecting similar endurance times. Conversely, some authors utilized training protocols with the same absolute intensity, which then can represent a higher stimulus in hypoxic conditions.

Bigard et al. (1991) observed a greater increase in C/F ratio in the plantaris, extensor digitorum longus and soleus muscles of rats housed and trained in a hypobaric chamber at an altitude equivalent to 4,000 m compared to animals trained under normoxic conditions (Bigard et al., 1991). Yet, in the same study C/F values were not different when comparing sedentary normoxic and hypoxic animals, suggesting that hypoxia alone is insufficient to promote muscle angiogenesis. Similarly, Olfert et al. (2001) trained rats for 8 weeks under ambient hypoxia (FiO_2 0.12) or normoxia. The C/F ratio was only increased in muscles from animals trained under hypoxia (Olfert et al., 2001).

Interestingly, Vogt et al. (2001) have also evaluated the effect of exercise intensity during hypoxia training in human subjects (Vogt et al., 2001). They reported a significant increase in capillarization only in the vastus lateralis muscles of subjects trained at high intensity for 12 weeks under hypoxia (simulated altitude of 3,850m). Training under normoxia, even at high intensity, did not improve significantly muscle capillarization.

Overall, these laboratory and well-controlled studies indicate a greater angio-adaptive response of the skeletal muscle tissue when exercise training is performed in ambient hypoxia. This reflects

into a higher level of muscle capillarization. Yet, it remains unknow whether muscle angio-adaptation occurs faster.

Field studies in humans for prolonged sojourn to altitude focusing on exercise and muscle angiogenic activity have provided mixed results. Mizuno et al. (1990) reported a significant increase in the triceps C/F of competitive cross-country skiers after 2 weeks of training at an altitude of 2,700m (Mizuno et al., 1990). However, because of the absence of proper control groups (sedentary and trained subjects in normoxia and hypoxia) it cannot really be discerned that these results were simply a response to exercise training. No change in C/F ratio was described in the rectus femoris or biceps brachii of climbers regularly engaged into intense climbing, walking, carrying activities at altitudes above 5,250 m (Levett et al., 2012; Mizuno et al., 2008).

Finally, the use of exercise training in hypoxia for improving certain health outcomes (biomechanical limitations, obesity, hypertension, ageing) is also gaining a high interest (Burtscher et al., 2004; Camacho-Cardenosa et al., 2020, 2019, 2018; Jung et al., 2021; Kong et al., 2017; Millet et al., 2016; Pramsohler et al., 2017; Ramos-Campo et al., 2019; Verges et al., 2015; Wiesner et al., 2010). In regard to skeletal muscle blood flow, capillarization and angio-adaptation, we and others have shown in rodent models and human patients that exercise interventions could represent a powerful therapeutic avenue to prevent, delay or improve alterations in chronic conditions such as peripheral limb ischemia, diabetes, obesity, chronic obstructive pulmonary diseases (Aiken et al., 2019; Amouzou et al., 2016; Armstrong et al., 1986; Gardner and Poehlman, 1995; Gouzi et al., 2013; Hudlicka et al., 1994; McDermott et al., 2009; Roudier et al., 2009). Yet, to the best of our knowledge the impact of hypoxia training as a therapeutic or preventive approach specifically for conditions affecting muscle capillarization and angio-adaptive activity remains to be investigated.

Muscle capillarization, exercise training and ambient hypoxia: Key points.

- Prolonged exposure to hypoxia can improve skeletal muscle capillary density likely because of myofiber atrophy. Yet the implication of a true angiogenic response cannot be ruled out.
- Combining training and ambient hypoxia can result in a higher muscle angiogenic response than training alone. Yet whether it could also result in an earlier response remains unknown.
- The existing literature obviously shows non-negligible discrepancy imputable sometimes to a lack of proper controls in early studies and to uncontrolled confounding factors such as activity level and training status, cold exposure, restriction in calory intake, animal growth.

Skeletal muscle capillarization in high-altitude native populations.

As mentioned previously, a classical idea is that prolonged exposure of lowlanders to high altitude could result in increased skeletal muscle CD often attributed to myofiber atrophy. Could this represent a phenotypic transition towards highlanders' muscles (Gilbert-Kawai et al., 2014)?

Interestingly, CD measured in highlanders' muscles do not seem to differ much from lowlanders. Kayser et al. (1991) have compared the CD from Tibetan Sherpas' muscles with average CD values from sedentary lowlanders or active climbers before and after a high altitude expedition (Kayser et al., 1991). The average CD was significantly higher (+20%) in Sherpas' muscles than in sedentary lowlanders' muscles. However, the training status of the subjects was not taken into consideration. In fact, there was no difference between Sherpas and active climbers before expedition, and a trend for a lower CD (-13%) was even observed in Sherpas' muscles when compared with post-expedition lowlander climbers' muscles (Kayser et al., 1991). It is also important to note that the CD values measured in Sherpas' muscles in this study (Kayser et al., 1991) were compared with CD obtained from different studies (Hoppeler et al., 1990b, 1973; Oelz et al., 1986). In another study, Kayser et al. (1996) have compared muscles from second-generation Tibetans living at low altitude with Nepalese controls: No difference was observed for muscle CD despite smaller fibers in Tibetans (Kayser et al., 1996).

In an interesting study, Lundby et al. (2004) have compared muscle CD from Aymara subjects, who live permanently around 3,800-4,100m altitude in Bolivia, with CD values from lowlanders' muscles before and an 8-weeks sojourn at 4,100 m (Lundby, 2004). Aymara muscle CD was 12% (trend) and 15% (significant) lower than lowlanders' CD respectively measured before and after the altitude sojourn. This apparent lower CD is intriguing given that high-altitude natives had 25-

30% smaller myofibers. The C/F ratio was in fact significantly 40% lower in Aymara's muscles compared to lowlanders.

Altogether, these results suggest that skeletal muscles from high-altitude residents, although presenting smaller fibers, do not have a higher CD than lowlanders' muscles. The observation of a much lower C/F ratio in high-altitude natives reminds the few studies reporting a tendency for a decreased C/F in lowlanders following a prolonged exposure to hypoxia (Green et al., 1989; Hoppeler et al., 1990b; Lundby, 2004; MacDOUGALL et al., 1991). It is then tempting to conclude this section by questioning whether a long-term angio-adaptation of the skeletal muscle to hypoxia could in fact result in some capillary regression. Angio-adaptation ensures an optimal match between the muscle capillarization and the metabolic needs of myofibers. If prolonged hypoxia results in an increased CD and smaller myofibers with decreased mitochondria volume density (Favier et al., 2015; Horscroft et al., 2017; Horscroft and Murray, 2014; Murray and Horscroft, 2016), some existing capillaries might at some point become unnecessary.

Molecular aspect of skeletal muscle angio-adaptive responses to hypoxia.

The skeletal muscle capillary network possesses a remarkable plasticity and whether capillaries regress, stabilize or grow in response to a stimulus is largely determined by an intricate balance of pro- and anti-angiogenic molecules (Breen et al., 2008; Egginton and Birot, 2014; Hoppeler, 1999; Olfert et al., 2015; Olfert and Birot, 2011). Among the plethora of angio-adaptive molecules described in the literature, the use of transgenic animal models identified two of them as key regulators of skeletal muscle angio-adaptation: The pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A) (Baum et al., 2017; Olfert et al., 2010, 2009; Tang et al., 2004); and the anti-angiogenic thrombospondin-1 (THBS-1) (Malek and Olfert, 2009; Slopack et al., 2014). Different methodological approaches have been used, some targeting VEGF-A in the whole muscle tissue, some targeting it specifically in myofibers (Baum et al., 2017; Breen et al., 2008; Malek and Olfert, 2009; Olfert et al., 2010; Tang et al., 2004). VEGF-A deletion results in (1) decreased basal level of muscle capillarization, (2) blunted exercise-induced angiogenic response, (3) severely reduced exercise capacity. Targeting the anti-angiogenic THBS-1 has somehow opposite consequences: Better vascularized muscles and greater exercise capacity (Breen et al., 2008; Malek and Olfert, 2009; Olfert et al., 2015, 2009). In the context of the present review on skeletal muscle angio-

adaptive responses to hypoxia, it is therefore important to recapitulate the response of VEGF-A and THBS-1 to exercise and ambient altitude (Figure 5).

Most of studies in exercise and altitude physiology have essentially focused on measuring VEGF-A mRNA and protein expression levels. However, how hypoxia can influence VEGF-A expression and activity is in fact very complex (Breen et al., 2008; Semenza, 2001). The *VEGFA* gene can be considered as a hypoxia-sensitive gene since it possesses several Hypoxia Responsive Elements (HRE) recognized by the transcription factor HIF-1. Under hypoxic conditions, the stabilization of HIF-1α and HIF-1 enhanced transcriptional activity lead to increased VEGF-A mRNA levels (Hoppeler et al., 2003). VEGF-A mRNA can then be stabilized via interaction between its 3' untranslated region and the Human antigen R (HuR protein) (Amadio et al., 2008; Morfoisse et al., 2015; Osera et al., 2015). Interestingly, Tang et al. (2002) have described such interaction between HuR and VEGF-A mRNA in rat ischemic gastrocnemius muscles (Tang et al., 2002). VEGF-A mRNA translation into proteins can also be affected by hypoxia. Indeed, whereas the classical mechanism of mRNA cap-dependent translation can be inhibited under hypoxia, some proteins such as VEGF-A can still be translated by using an alternative translational mechanism where cap-independent translation is initiated via internal ribosome entry sites (IRESs) (Huez et al., 1998; Miller et al., 1998; Stein et al., 1998). The endoplasmic reticulum chaperone Oxygen Regulated Protein-150 (ORP-150) can finally facilitate VEGF-A protein secretion, and an increase in ORP-150 has been reported in rat plantaris muscles following an acute bout of running exercise (Birot et al., 2003; Ozawa et al., 2001a, 2001b). Finally, hypoxia can also stimulate the expression of VEGF-A receptors (Gerber et al., 1997).

A stimulatory effect of exercise on VEGF-A expression has been well described in human and rodent studies. Both mRNA and protein levels are transiently increased after one bout of exercise (Aiken et al., 2016; Ameln et al., 2005; Birot et al., 2003; Breen et al., 2008, 1996; Croley et al., 2005; Gavin et al., 2007, 2006; Gustafsson et al., 2005, 2002; Gustafsson and Kraus, 2001; Hoppeler and Vogt, 2001; Roudier et al., 2012). A study from Breen et al. (1996) has shown that increases in mRNA in rat skeletal muscle were higher and lasted longer when exercise was performed at a higher intensity (Breen et al., 1996). An attenuation of VEGF-A mRNA responsiveness to exercise stimulus has be observed after short-term (few days) training (Gavin and Wagner, 2001; Kraus et al., 2004; Olfert et al., 2001; Vogt et al., 2001).

The effect of acute or chronic exposure to ambient hypoxia itself on VEGF-A expression are less conclusive. Most of the studies that have assessed VEGF-A expression in the muscle tissue have measured mRNA levels only, whereas the protein was essentially quantified in the plasma or serum by ELISA (Asano et al., 1998; Bahtiyar et al., 2007; Boos et al., 2018; Brinkmann et al., 2017; Ding et al., 2012; Dorward et al., 2007; Gunga et al., 2003, 1999; Hanaoka et al., 2003; Kasai et al., 2019; Kasperska and Zembron-Lacny, 2020; Morici et al., 2013; Oltmanns et al., 2006; Schobersberger et al., 2000). Results from the literature remain largely inconsistent, reporting some increase in mRNA or protein (Breen et al., 1996; Gavin et al., 2006), some attenuation (Boos et al., 2018; Morici et al., 2013; Olfert et al., 2001; Oltmanns et al., 2006), or no change (Bahtiyar et al., 2007; Gunga et al., 2003; Lundby, 2004). Additionally, circulating VEGF-A levels measured by ELISA do not necessarily reflect skeletal muscle VEGF-A production.

Finally, some studies have analyzed VEGF-A responsiveness when combining exercise and ambient hypoxia (Asano et al., 1998; Breen et al., 1996; Brocherie et al., 2018; Gunga et al., 2003; Kasperska and Zembron-Lacny, 2020; Nagahisa et al., 2016; Olfert et al., 2001; Vogt et al., 2001; Zoll et al., 2006). Breen et al. (1996) have shown that VEGF-A mRNA expression in rat skeletal muscle was higher and last longer when exercise was performed in ambient hypoxia compared to a normoxic environment (Breen et al., 1996). Brocherie et al. (2018) have compared the effect of different modalities of exercise training combined with hypoxia exposure (Brocherie et al., 2018). VEGF-A mRNA levels were only increased in human skeletal muscle in their model of Live High-Train Low and High (LHTLH) that combined passive exposure to ambient hypoxia and exercise training session at maximal intensity under hypoxia, and no change in VEGF-A was observed in the other two models (Live High-Train Low, LHTL, and Live Low-Train Low, LLTL).This study is in line with previous results reported by Vogt and colleagues (2001) who analyzed VEGF-A mRNA expression in response to a training program realized either at high or low intensity and either in normoxia or hypoxia (6 weeks at an equivalent of 3,850 m) (Vogt et al., 2001). Whereas training at high intensity in normoxia and training at low intensity in hypoxia only led to a trend for an increase in VEGF-A mRNA (respectively, +13 and +17%), only the combination of training at high intensity in hypoxia resulted in significant increase (+72%). These studies support the idea that combining exercise and ambient hypoxia could exacerbate the angio-adaptive response of the skeletal muscle tissue. If the duration, nature and intensity of the training program are key parameters, all these studies also point out the importance of the duration and intensity of hypoxia

exposure. This has recently led to the notion of hypoxic dose (D'Hulst and Deldicque, 2017; Girard et al., 2020; Lundby et al., 2012, 2009; Millet et al., 2017).

Thrombospondin-1 has been identified as a potent anti-angiogenic factor in the skeletal muscle tissue (Hellsten and Hoier, 2014; Malek and Olfert, 2009). Similar to VEGF-A, one bout of exercise stimulates the expression of THBS1mRNA and protein in skeletal muscle (Malek and Olfert, 2009; Olfert et al., 2006; Slopack et al., 2014) whereas short-term training is accompanied by a progressive loss of responsiveness of THBS1 to exercise stimulus (Hoier et al., 2020; Olfert et al., 2006; Slopack et al., 2014) (Figure 5). This responsiveness however appears to be restored with long-term training (Hoier et al., 2012; Olfert et al., 2006; Slopack et al., 2014). The decreased responsiveness of THBS1 during training could in fact contribute to shifting the skeletal muscle angio-adaptive balance towards its pro-angiogenic side, reflected at the tissue level by a pro-angiogenic microenvironment prone to capillary formation. This has led to the hypothesis that exercise-induced angiogenesis might in fact be more controlled by a decrease in anti-angiogenic factors rather than an increase in pro-angiogenic ones (Hellsten and Hoier, 2014; Olfert, 2016; Olfert et al., 2015; Olfert and Birot, 2011). This could apply to capillary regression, as during detraining or muscle hypokinesia, with an increase expression of anti-angiogenic factors shifting the angio-adaptive balance the opposite way (Kishlyansky et al., 2010; Olenich et al., 2014; Olfert and Birot, 2011; Roudier et al., 2010, 2009).

Long-term training does not seem to alter the basal expression level of THBS1 in rodent and human healthy skeletal muscles (Gliemann et al., 2015; Hoier et al., 2012; Olfert et al., 2006) although Yoshioka et al. (2003) have reported higher THBS1 gene expression in skeletal muscles from endurance athletes compared to sedentary subjects (Yoshioka et al., 2003). Interestingly, Gouzi et al. (2013) have shown that muscle THBS1 protein levels could be reduced in response to prolonged training in patients with chronic obstructive pulmonary disease (Gouzi et al., 2013). This reinforces the idea of using exercise training as a therapeutic avenue for clinical conditions with skeletal muscle microvascular alterations. THBS1 expression was indeed found to be increased in rodent skeletal muscles in the context of diabetes, pre-diabetes and hindlimb ischemia (Aiken et al., 2019; Dunford et al., 2017; Kivelä et al., 2008, 2006; Roudier et al., 2013).

The effect of hypoxia alone on THBS1 expression has provided mixed results essentially from *in-vitro* experiments. Phelan et al., (1998) have described an increased expression of THBS1 mRNA

and protein in endothelial cells exposed to severe hypoxia (1%O_2) (Phelan et al., 1998). Conversely, Yadav et al., (2014) have observed a decrease in THBS1 expression in differentiated murine C2C12 myotubes in response to hypoxia (Yadav et al., 2014). Finally, THBS1 expression was found to be increased both in rodent and human ischemic skeletal muscles (Roudier et al., 2013).

To our knowledge, there is very limited data regarding THBS1 expression in skeletal muscle when combining exercise stimulus and ambient hypoxia. Olfert et al. (2006) have analyzed THBS1 mRNA expression in skeletal muscles from rats kept sedentary or enrolled into a 8-weeks endurance running program (Olfert et al., 2006). At the end of the training program, animals were performing one bout of intense running exercise. Data suggests that endurance training did not alter the basal expression level of THBS1. However, chronic hypoxia exposure resulted in lower basal expression level (-44%) as well as THBS-1 expression in response to one bout of exercise (-48%).

As a conclusion to this section, it is important to keep in mind that VEGF-A and THBS1 are only two members of the large family of angio-adaptive molecules susceptible to influence the skeletal muscle angio-adaptive responses to exercise and ambient hypoxia. The expression levels of some other angio-adaptive molecules have been measured in skeletal muscle tissue in the context of exposure to ambient hypoxia, such as basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor-β (TGF-β), VEGF receptors, leptin (Breen et al., 1996; Morici et al., 2013; Olfert et al., 2001; Patitucci et al., 2009). However, these measurements remain very anectodical, and more research in this area is needed.

Here, we have also essentially reviewed VEGF-A and THBS1 expression levels, which do not reflect the functionality of these molecules, their interaction between each other's and their receptors.

As illustrated in Figure 5, the angio-adaptive response to a given stimulus is a complex and dynamic process that involves molecular, cellular, and tissular responses. There is for example a lack of knowledge regarding cross-talks between muscle cells. For instance, how angio-adaptive signals originating from a contractile myofiber will stimulate neighboring endothelial cells to proliferate and migrate to form new capillaries.

Ideally, evaluating the muscle angio-adaptive response to hypoxia should be integrative. For example, an absence of significant change in capillarization does not rule out any angio-adaptive

responses at a cellular or molecular level. Finally, the interpretation of results from the literature is very complex with a certain discrepancy between studies. Such divergence can obviously have several origins: Animal versus human studies; healthy versus pathological conditions; training status; exercise protocols; hypoxia level and duration; confounding environmental stressors (cold, air pollution); time of sample collection; methodology (northern blotting, qPCR, western blotting, ELISA, histochemistry).

The skeletal muscle as an endocrine organ and the role of angio-adaptive myokines.

As previously mentioned, many studies studying angiogenic responses to hypoxia and exercise have quantified circulating VEGF-A protein levels by ELISA. Several studies have aimed to link changes in circulating VEGF-A levels with the susceptibility to develop acute or chronic mountain sickness (Dorward et al., 2007; Espinoza et al., 2014; Nilles et al., 2009; Schommer et al., 2011; Tissot van Patot et al., 2005). Regarding THBS1, this anti-angiogenic molecule seems to be involved in the pathophysiology of hypoxia-induced pulmonary hypertension and right ventricular hypertrophy (Bauer et al., 2012; Ochoa et al., 2010; Rogers et al., 2017). Kaiser et al. (2016) have reported elevated serum THBS1 levels and strong correlations of serum THBS1 to mean pulmonary artery pressure and pulmonary vascular resistance in patients suffering from pulmonary hypertension (Kaiser et al., 2016).

Interestingly, many circulating angio-adaptive molecules, either pro- or anti-angiogenic, could therefore represent valuable biomarkers to evaluate for example the response of athletes to a specific training program in hypoxia or the impact of a therapeutic exercise intervention in a group of patients. More research in identifying the patho-physiological relevance of circulating angio-adaptive biomarkers would be exciting and essential.

The quantification of circulating angio-adaptive molecules also points out the role of the skeletal muscle as an endocrine organ secreting myokines to act on distant organs such as bones, brain, fat, and liver (Delezie and Handschin, 2018; Fabel et al., 2003; Gomarasca et al., 2020; Kim et al., 2019; Schnyder and Handschin, 2015). Fabel et al. (2003) have demonstrated that peripherally-produced VEGF-A seems necessary for running-induced improvements in hippocampal neurogenesis (Fabel et al., 2003). Rich et al. (2017) confirmed that VEGF-A produced by skeletal myofibers plays an important role in hippocampal neurogenesis (Rich et al., 2017). Interestingly, it was also suggested that VEGF-A meditated neurogenesis could provide a neuroprotective effect

and could be essential for attenuating decrements to cognitive function experienced with ambient hypoxia during high altitude exposure (Koester-Hegmann et al., 2019).

Molecular aspect of skeletal muscle angio-adaptive responses to hypoxia: Key Points.

- Skeletal muscle angio-adaptation is a complex and dynamic process combining molecular and cellular responses that will ultimately alter muscle capillarization.
- Several angio-adaptive molecules have been described in the skeletal muscle tissue in the context of exercise-induced angio-adaptation. Yet, their characterization in the context of exposure to ambient hypoxia remains largely unknown.
- There is a current lack of consensus in the literature largely due to confounding experimental variables regarding the expression of muscle VEGF-A and circulating VEGF-A in response to ambient hypoxia alone and to training in hypoxia.
- Anti-angiogenic molecules, such as THBS1, could in fact impact skeletal muscle capillarization to a greater extent than VEGF-A. Their contribution in skeletal muscle angio-adaptive responses to exercise and hypoxia is however understudied.
- The patho-physiological relevance of circulating angio-adaptive molecules as biomarkers remains poorly documented.
- The role of the skeletal muscle tissue as an endocrine organ secreting angio-adaptive myokines to act on distant organs in the context of exercise and hypoxia is an exciting research avenue.

Skeletal muscle angio-adaptive responses to hypoxia: Do normobaric and hypobaric conditions differ?

The research studies discussed in our review were conducted in three types of environments: Field experiments with real altitude exposure versus hypoxic chambers simulating altitude. Chambers where the barometric pressure and partial oxygen pressure are decreased represent a hypobaric environment, closer to the physics of real altitude, whereas normobaric chambers generate a hypoxic environment by decreasing the fraction of oxygen in the inspired air. Whether normobaric and hypobaric hypoxia are equivalent and then interchangeable has been an intense source of scientific debate, particularly with regards to their impact on exercise performance and various physiological parameters. Systematic reviews and “points-counterpoints” discussions have

however not enable researchers to reach any consensus (Coppel et al., 2015; Debevec and Millet, 2014; DiPasquale et al., 2016; Faiss et al., 2013; Girard et al., 2012; Millet et al., 2013, 2012a, 2012b; Mounier and Brugniaux, 2012a, 2012b; Richalet, 2020a, 2020b; Richard et al., 2014). Discrepancy between studies can be attributable to many cofounding factors: Different degrees of hypoxia, themselves determined either by barometric pressure, inspired PO_2, oxygen fraction; seasonal and geographical differences in barometric pressure; air temperature and humidity; additional environmental stressors such as cold exposure; duration of exposure; presence or not of exercise interventions, and if so different exercise protocols; characteristics of subjects; animal versus human studies.

To the best of our knowledge, whether normobaric and hypobaric hypoxia could differently affect the angio-adaptive responses of the skeletal muscle has never been investigated. We have therefore revisited the original research studies cited in our review that analyzed CD, C/F, or the expression levels of angio-adaptive molecules (mRNA or protein levels, tissue or circulating levels) in response to ambient hypoxia exposure. This represents 49 original studies (Figure 6). We separated them accordingly to the hypoxic environment used: Field studies (hypobaric), hypobaric chambers, combined field and hypobaric chambers (both hypobaric environments), and normobaric chambers. Studies involving physical activity (comparing active versus less active subjects, including one bout of exercise or prolonged training) were considered only if they possessed all required control groups enabling the evaluation of the impact of ambient hypoxia *per se*. We also distinguished studies conducted in animal models or human subjects. Finally, we determined in each category the percentage of studies reporting significant changes (increase or decrease) in capillarization (CD and/or C/F) and expression of angio-adaptive molecules.

Unfortunately, the information presented in Figure 6 does not really help in answering the question on whether normobaric and hypobaric hypoxia could differently affect the angio-adaptive responses of the skeletal muscle. Indeed, when considering only the studies reporting significant changes in capillarization, a distinction could be made between those conducted in normobaric chambers (70% presenting significant changes) versus studies run in a hypobaric environment (33% for field studies, 56% for hypobaric chambers, and 44% for combined hypobaric environments). However, no such distinction could be made when considering only the studies reporting significant changes in angio-adaptive molecules (67% for field studies, 70% for combined hypobaric environments, and 77% for studies conducted in normobaric chambers).

Finally, when looking at "overall" changes (capillarization and angio-adaptive molecules), it seems that a larger proportion of studies conducted in hypoxic chambers, whether normobaric or hypobaric, report significant changes (respectively 74% and 67%) compared to field studies (53%). As mentioned earlier, a possible explanation could be that field studies often present cofounding factors. Another interesting observation from Figure 6 is that these conditionings in hypoxic chambers were mainly performed in animal models whereas field studies were essentially involving human subjects (respectively 33% and 48% of human studies involving hypobaric and normobaric chambers versus 88% for field studies). Similar durations of exposure to hypoxia will obviously not represent the same proportion of a lifespan between rodents and humans.

Based on this analysis, we do not believe that any strong consensus can be established regarding the impact of hypobaric versus normobaric hypoxia on skeletal muscle angio-adaptive responses.

Conclusion.

Hypoxia, defined as a reduction of oxygen availability can occur in the skeletal muscle tissue of an individual exposed to ambient hypoxia as well as during physical exercise if the oxygen supply to contracting myofibers cannot match their increased metabolic needs. The superimposition of these two stressors, ambient hypoxia exposure and exercise-induced local hypoxia, can lead to an exacerbation of the hypoxic stress experienced by the skeletal muscle. The capillary-to-myofiber interface serves as the site for the exchange of oxygen, nutrients, metabolic heat, and waste between the blood and myofibers. As such, the capillary microvasculature is tightly related to the functional capacity of the skeletal muscle. The capillary microvasculature is a highly adaptive tissue with remarkably plasticity that can grow or regress to various physiological, pathological, and environmental stressors, a process named angio-adaptation. Skeletal muscle angio-adaptation involves complex and dynamic molecular and cellular responses. Given the relevance of skeletal muscle angio-adaptation in response to hypoxia to mountaineers, athletes, and clinical populations, this review aimed to delineate the existing literature and identify current gaps in the knowledge of this field of environmental and exercise physiology (Figure 7).

FIGURE 3

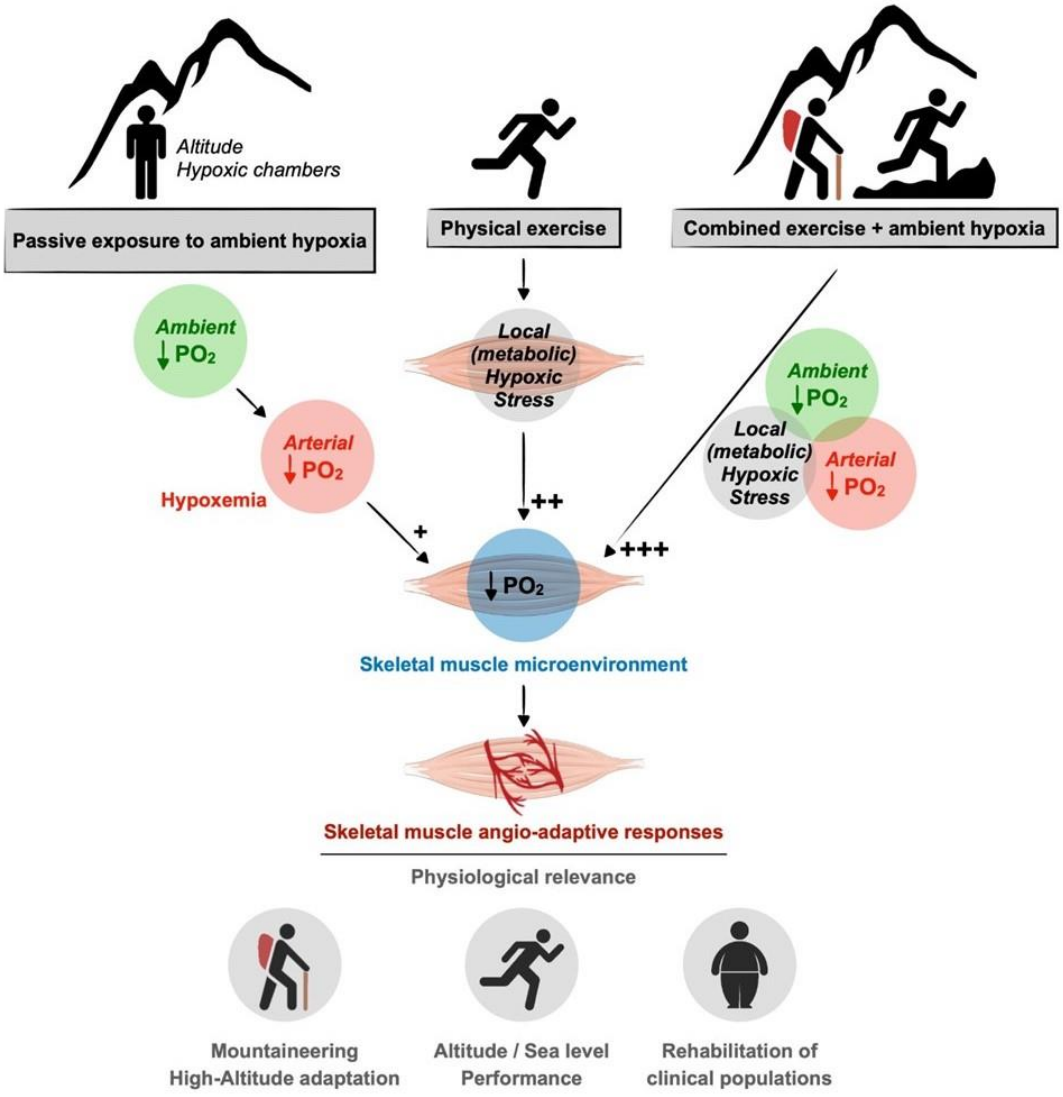

Altitude
Hypoxic chambers
Passive exposure to ambient hypoxia
Physical exercise
Combined exercise + ambient hypoxia
Ambient ↓PO2
Arterial ↓PO2
Hypoxemia
Local (metabolic) Hypoxic Stress
++
+
+++
↓PO2
Skeletal muscle microenvironment
Skeletal muscle angio-adaptive responses
Physiological relevance
Mountaineering
High-Altitude adaptation
Altitude / Sea level
Performance
Rehabilitation of
clinical populations

Figure 3. Contribution of ambient hypoxia and exercise to induce a hypoxic stress in skeletal muscle. A drop in the partial pressure of oxygen (PO_2) in the muscle tissue can result from a decrease in ambient PO_2 (e.g., altitude) as well as an unbalanced between blood supply to the muscle cells and their increase metabolic needs during exercise. In response to muscle hypoxia angio-adaptive responses will take place to maintain an optimal oxygen supply to myofibers and to preserve muscle function. This is of particular relevance in the context of mountaineering activity as well as exercise training strategies using ambient hypoxia for athletes or clinical populations. As discussed in the text, the impact of ambient hypoxia on skeletal muscle PO_2 seems rather modest (+) compared to the impact of exercise in generating a local hypoxic stress (++). The combination of ambient hypoxia exposure and exercise might exacerbate this hypoxic stress (+++).

FIGURE 4

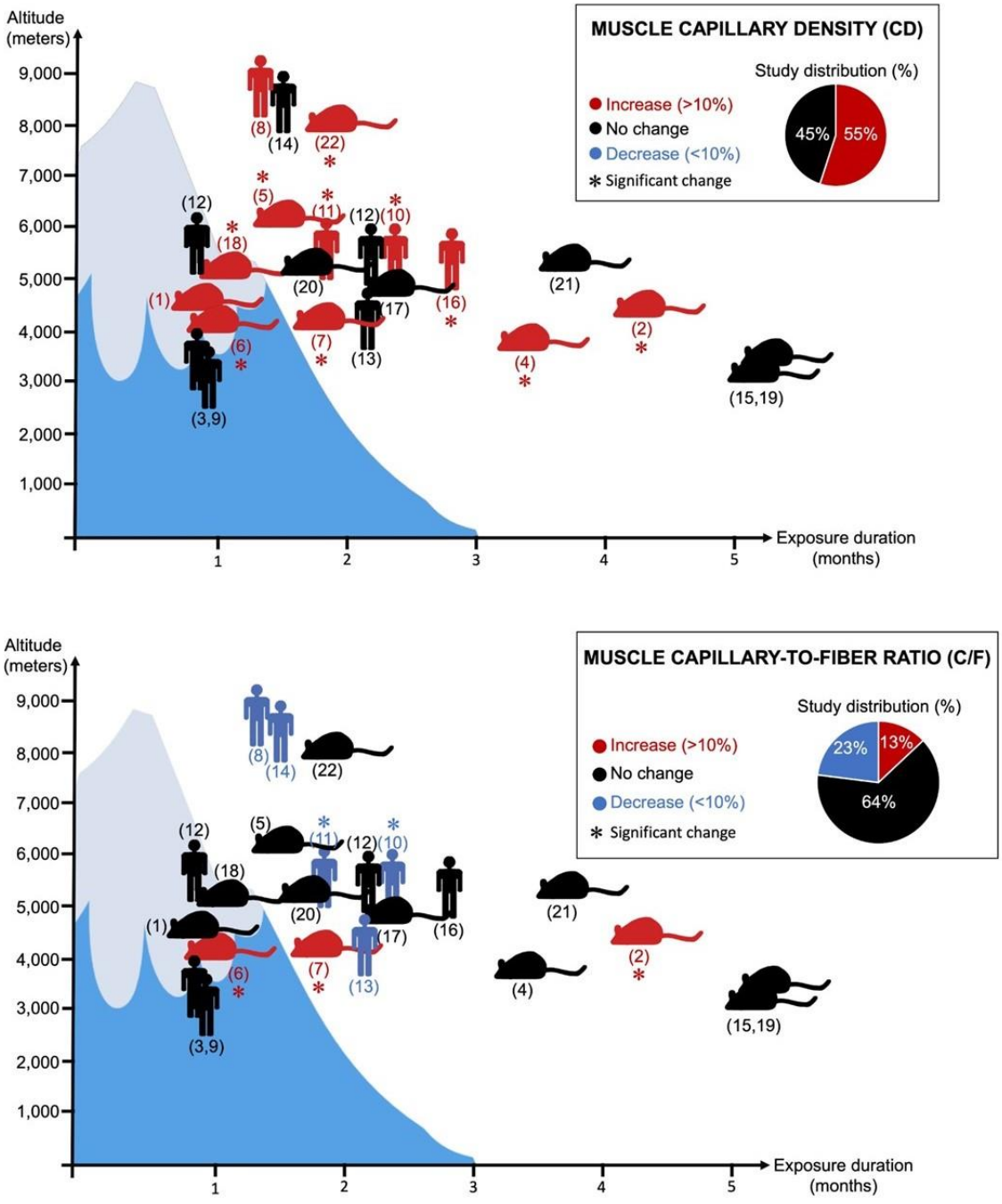

MUSCLE CAPILLARY DENSITY (CD)
Study distribution (%)
Increase (>10%)
No change
Decrease (<10%)
Significant change
45%
55%
MUSCLE CAPILLARY-TO-FIBER RATIO (C/F)
Study distribution (%)
Increase (>10%)
No change
Decrease (<10%)
Significant change
23%
13%
64%
Altitude (meters)
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
Exposure duration (months)
1
2
3
4
5
(1)
(2)
(3,9)
(4)
(5)
(6)
(7)
(8)
(10)
(11)
(12)
(13)
(14)
(15,19)
(16)
(17)
(18)
(20)
(21)
(22)

Figure 4. Twenty-two research articles analyzing changes in skeletal muscle capillary density (CD) (Panel A) or capillary-to-fiber ratio (C/F) (Panel B) in response to prolonged exposure to ambient hypoxia were surveyed. (🧍) indicates human studies. (🐁) indicates the use of animal models (not restricted to rodents). (*) indicates significant finding. Red coloration indicates an increase in magnitude by 10% or greater. Blue coloration indicates a decrease in magnitude by 10% or greater. Black coloration indicates no change. Y- and X-axis respectively reflect the level of altitude (real or simulated) and the duration exposure. Corresponding references: **(1)** Banchero et al., 1976; **(2)** Banchero et al., 1985; **(3)** Basset et al., 2006 ; **(4)** Bigard et al., 1991 ; **(5)** Cassin et al., 1971 ; **(6)** Deveci et al., 2001 ; **(7)** Deveci et al., 2002 ; **(8)** Green et al., 1989 ; **(9)** Green et al., 1992 ; **(10)** Hoppeler et al., 1990a ; **(11)** Hoppeler et al., 1990b ; **(12)** Levett et al., 2012 ; **(13)** Lundby et al., 2004 ; **(14)** MacDougall et al., 1991 ; **(15)** Mathieu-Costello & Agey, 1997 ; **(16)** Mizuno et al., 2008 ; **(17)** Olfert et al., 2001 ; **(18)** Panisello et al., 2008 ; **(19)** Poole & Mathieu-Costello, 1989 ; **(20)** Sillau & Banchero, 1977 ; **(21)** Sillau et al., 1980b ; **(22)** van Ekeren et al., 1992.

FIGURE 5


Skeletal muscle angio-adaptive responses to exercise and ambient hypoxia
Acute phase (hrs to 24-28h)
Short-term phase (5-10 days)
Long-term phase (weeks)
Timeline
Illustration of the dynamic and multi-level (molecular, cellular, tissular) aspect of skeletal muscle angio-adaptation
Angio-adaptive molecular and cellular responses
Production and release of pro- and anti-angiogenic signals locally in the muscle tissue and in the circulation.
VEGF-A
THBS1
Increased migratory capacity of microvascular endothelial cells
Angio-adaptive molecular and cellular responses
Enzymatic degradation of extracellular matrix (sprouting angiogenesis).
Active phase of endothelial cells proliferation and migration to assembly into vascular tubes (sprouting angiogenesis).
Angio-adaptive molecular, cellular, and tissular responses
Restoration of anti-angiogenic factor expression (angiogenic "brake")
Endothelial cell "stabilization"
Pericyte recruitment
+/- changes in capillary density
+/- changes in capillary-to-fiber ratio
+/- changes in tortuosity
Exercise
VEGF-A
Increased expression
Increased expression +/- some decreased responsiveness
Unclear (higher basal level not always observed)
THBS1
Increased expression
Decreased responsiveness
Restoration of responsiveness
CD
Unchanged
Unchanged
Increased
C/F
Unchanged
Unchanged
Increased
Ambient Hypoxia
VEGF-A
Increased expression
Unknown / Unclear
Unclear, some studies reporting an attenuation of expression
THBS1
Decreased in vitro in mouse cells
Unknown / Unclear
Decreased
CD
Unchanged
Unchanged
Often reported as increased but should be revisited
C/F
Unchanged
Unchanged
Often reported as unchanged but should be revisited
Combined
VEGF-A
Increased expression
Unknown / Unclear
Increased or unchanged
THBS1
Increased expression
Unknown / Unclear
Unknown
CD
Unchanged
Unchanged
Increased
C/F
Unchanged
Unchanged
Increased

Figure 5. Skeletal muscle angio-adaptation is a complex and dynamic process in which much earlier molecular and cellular events lead to eventual changes observed at the tissular level through modifications in the skeletal muscle's capillarization. In response to a given stimulus, whether it be the growth, regression, or stabilization of skeletal muscle capillaries, the process is dictated by an intricate balance of pro- and anti-angiogenic factors. Among the plethora of angio-adaptive factors two have been identified as central in the regulation of skeletal muscle capillarization: the pro-angiogenic VEGF-A; and the anti-angiogenic THBS1. Majority of current literature concerning hypoxic muscle angio-adaptation has focused on the regulation of VEGF-A and as such there are current gaps in knowledge concerning the hypoxic regulation of THBS1. Additionally, there is a lack of consensus regarding the impact of ambient hypoxia eliciting changes at the tissular level concerning CD and C/F.

FIGURE 6

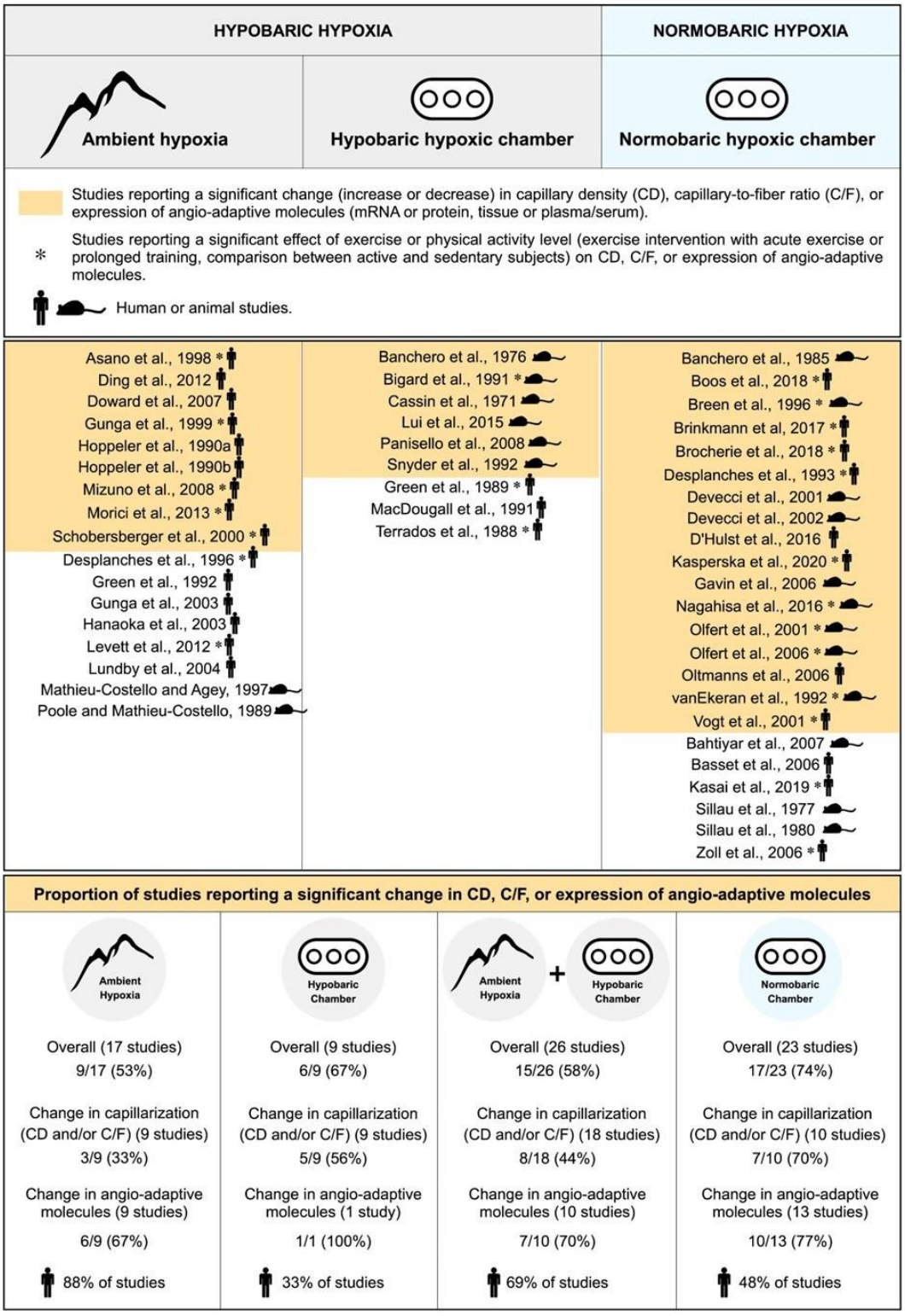

HYPOBARIC HYPOXIA
NORMOBARIC HYPOXIA
Ambient hypoxia
Hypobaric hypoxic chamber
Normobaric hypoxic chamber
Studies reporting a significant change (increase or decrease) in capillary density (CD), capillary-to-fiber ratio (C/F), or expression of angio-adaptive molecules (mRNA or protein, tissue or plasma/serum).
* Studies reporting a significant effect of exercise or physical activity level (exercise intervention with acute exercise or prolonged training, comparison between active and sedentary subjects) on CD, C/F, or expression of angio-adaptive molecules.
Human or animal studies.
Asano et al., 1998 *
Ding et al., 2012
Doward et al., 2007
Gunga et al., 1999 *
Hoppeler et al., 1990a
Hoppeler et al., 1990b
Mizuno et al., 2008 *
Morici et al., 2013 *
Schobersberger et al., 2000 *
Desplanches et al., 1996 *
Green et al., 1992
Gunga et al., 2003
Hanaoka et al., 2003
Levett et al., 2012 *
Lundby et al., 2004
Mathieu-Costello and Agey, 1997
Poole and Mathieu-Costello, 1989
Banchero et al., 1976
Bigard et al., 1991 *
Cassin et al., 1971
Lui et al., 2015
Panisello et al., 2008
Snyder et al., 1992
Green et al., 1989 *
MacDougall et al., 1991
Terrados et al., 1988 *
Banchero et al., 1985
Boos et al., 2018 *
Breen et al., 1996 *
Brinkmann et al, 2017 *
Brocherie et al., 2018 *
Desplanches et al., 1993 *
Devecci et al., 2001
Devecci et al., 2002
D'Hulst et al., 2016
Kasperska et al., 2020 *
Gavin et al., 2006
Nagahisa et al., 2016 *
Olfert et al., 2001 *
Olfert et al., 2006 *
Oltmanns et al., 2006
vanEkeran et al., 1992 *
Vogt et al., 2001 *
Bahtiyar et al., 2007
Basset et al., 2006
Kasai et al., 2019 *
Sillau et al., 1977
Sillau et al., 1980
Zoll et al., 2006 *
Proportion of studies reporting a significant change in CD, C/F, or expression of angio-adaptive molecules
Ambient Hypoxia
Hypobaric Chamber
Ambient Hypoxia + Hypobaric Chamber
Normobaric Chamber
Overall (17 studies)
9/17 (53%)
Change in capillarization (CD and/or C/F) (9 studies)
3/9 (33%)
Change in angio-adaptive molecules (9 studies)
6/9 (67%)
88% of studies
Overall (9 studies)
6/9 (67%)
Change in capillarization (CD and/or C/F) (9 studies)
5/9 (56%)
Change in angio-adaptive molecules (1 study)
1/1 (100%)
33% of studies
Overall (26 studies)
15/26 (58%)
Change in capillarization (CD and/or C/F) (18 studies)
8/18 (44%)
Change in angio-adaptive molecules (10 studies)
7/10 (70%)
69% of studies
Overall (23 studies)
17/23 (74%)
Change in capillarization (CD and/or C/F) (10 studies)
7/10 (70%)
Change in angio-adaptive molecules (13 studies)
10/13 (77%)
48% of studies

Figure 6. Characterization of the impact of hypobaric and normobaric hypoxia on skeletal muscle angio-adaptive responses. Original research studies cited in the review and analyzing capillary density (CD), capillary-to-fiber ratio (C/F), and expression levels of angio-adaptive molecules in skeletal muscle tissue in response to ambient hypoxia exposure were characterized based on the nature of their hypoxic environment: Hypobaric or normobaric. Studies reporting significant changes in capillarization (CD or C/F), in molecular expression, or in both ("Overall") are highlighted in colour. Studies involving a physical activity component are identified by an asterisk. The mouse and human silhouette symbols distinguish studies conducted in animal models or human subjects.

FIGURE 7

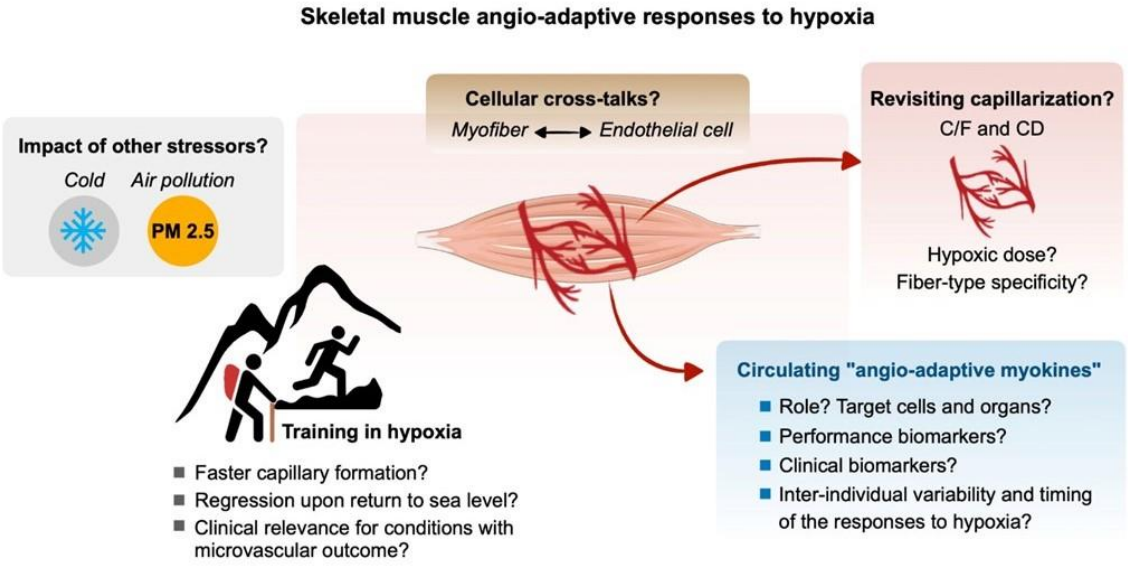

Skeletal muscle angio-adaptive responses to hypoxia
Impact of other stressors?
Cold
Air pollution
PM 2.5
Cellular cross-talks?
Myofiber
Endothelial cell
Revisiting capillarization?
C/F and CD
Hypoxic dose?
Fiber-type specificity?
Training in hypoxia
Faster capillary formation?
Regression upon return to sea level?
Clinical relevance for conditions with microvascular outcome?
Circulating "angio-adaptive myokines"
Role? Target cells and organs?
Performance biomarkers?
Clinical biomarkers?
Inter-individual variability and timing of the responses to hypoxia?

Figure 7. Gap of knowledge and future research directions for a better understanding of the molecular, cellular and tissular angio-adaptive responses of the skeletal muscle tissue to hypoxia, particularly in the context of ambient hypoxia and exercise-induced local hypoxia. Refer to the different text sections for details.

3. STUDY 2

Angiogenic move or angiostatic freeze? Evaluating the angio-adaptive impact of acute cold stress exposure on cultured murine skeletal muscle myotubes and endothelial cells.

Lemieux, P., Roudier, E., Birot, O., 2022. Angiogenic move or angiostatic freeze? Evaluating the angio-adaptive impact of acute cold stress exposure on cultured murine skeletal muscle myotubes and endothelial cells. Manuscript in preparation for submission to *Frontiers in Physiology*.

Lemieux, P., Roudier, E., Birot, O., 2022. Move or freeze? Elucidating the angiogenic relationship between mouse skeletal muscle endothelial cells and differentiated C2C12 myotubes. 18th International Biochemistry of Exercise Conference (IBEC), Toronto, Canada. May 25-28, 2022.

Author Contributions

Myself and my supervisor, Dr.Birot, have equally contributed to the design of experiments and the interpretation of data used in this study. Myself and Dr.Birot have equally contributed to the writing of the methods section. I have personally performed all the experiments, quantified all the data, written the results and discussion sections, created all figures, and written all figure legends. Dr.Birot and my second reader, Dr.Roudier, have equally contributed to harvesting muscle samples used for the ex-vivo muscle incubation assay (Figure 17, Supplemental Figure 4).

3.1. Abstract.

Skeletal muscle tissue adapts to various stressors with remarkable plasticity, cold-stress is no exception to eliciting these adaptations. Indeed, prolonged cold-stress exposure improves muscle capillarization. Skeletal muscle angio-adaptation refers to molecular and cellular processes that influence the remodeling of the muscle microvasculature. Two cell types are central to these processes, the myocyte representing a source of angiokines, and the skeletal muscle endothelial cell (SMECs), a target of these angiokines and the main constituent of muscle capillaries. Furthermore, cold-stress influences the expression and functionality of proteins and genes related to the microvasculature in the myocyte and whole muscle. It is largely unknown however the influence of cold-stress on skeletal muscle angio-adaptation with respect to myocyte-specific angiokines or endothelial cell angio-adaptive responses. Here, we used an *in-vitro* model to investigate the impact of cold-stress (28℃) on C2C12 myotubes or SMECs. Our objectives were to evaluate: (1) the impact of cold-stress on the expression of angiokines from C2C12 myotubes; (2) the indirect impact of cold-stress on SMECs migration via these C2C12-derived angiokines; (3) the effect of cold-stress on SMECs angio-adaptive responses (migration, proliferation, Vascular endothelial growth factor receptor-2 (VEGFR2) activation). Cold-stress reduced the expression of angiokines in C2C12 myotubes culture media irrespective if pro- or anti-angiogenic. SMECs exposed to cold-stress had an abrogated proliferative capacity. A reduced activation of VEGFR2 was also observed in cold-stressed SMECs despite a greater expression of VEGFR2. Finally, SMECs pre-conditioned to cold stress displayed an enhanced migratory response when migration was stimulated in re-warming conditions. Our findings suggest that cold-stress may be angiostatic overall. This is supported by the fact that cold-stress attenuated the capacity of the myocyte to release angiokines in addition to reducing SMECs proliferation and VEGFR2 activation. Cold-stress accompanied by re-warming however may be seen as an angiogenic stressor for SMECs. These results can question the efficacy for the use of cold-stress in sport-performance or therapeutic settings to improve muscle capillarization (i.e cold water immersion). Moreover, it would be intriguing to investigate in future works whether pre-cooling could be used as a strategy to prime endothelial cells for an enhanced angiogenic response.

3.2. Scientific context of the study.

Skeletal muscle displays a remarkable plasticity by adapting to physiological, pathological, and environmental conditions. In the context of high altitude, I have mentioned in **Study 1** that cold stress was reported as a potential confounding factor of ambient/environmental hypoxia, capable of affecting skeletal muscle angio-adaptation. Yet, in comparison to ambient hypoxia, the effect of cold stress on skeletal muscle angio-adaptation has been much less investigated.

From people working or exercising in cold environments, from low ambient temperatures to high winds to immersion in cold water, from therapeutic uses to an occupation hazard, cold stress is an environmental stressor that can be encountered in various circumstances. Furthermore, cold stress is unique in that it can be experienced differently from person to person based on individual circumstances and subjective evaluations. For example, when Dr. Mike Tipton stated, "a man in the cold is not necessarily a cold man", he was referring to the fact that despite being in a cold environment additional factors such as metabolic heat production, layers of clothing, anthropometric differences, sex, and fitness status may all contribute to how an individual subjectively experiences cold (The Physiological Society, 2016). Therefore, with cold stress having a high degree of individual subjectivity the question arises, how can the degree of cold stress be measured?

With respect to skeletal muscle, temperature provides a quantitative index for describing the degree of cold stress. In resting conditions, skeletal muscle temperature has been reported to be about 34-36℃, yet during instances of local muscle cooling can be reduced to as low as 18-22℃ (Barcroft and Edholm, 1946; Clarke et al., 1958). However, factors such as the muscle analyzed, or the depth of the thermal probe can influence muscle temperature measurements. Furthermore, whether cold stress is applied in the form of ambient air cooling or cold-water immersion can also affect the degree to which muscle temperature is reduced with water having a thermal conductivity that is 25x that of air (Castellani and Young, 2016).

Regarding skeletal muscle capillary remodelling, evaluating the impact of cold stress on angio-adaptive processes can be valuable for the following reasons. First, skeletal muscle functions as a thermogenic organ through both shivering and non-shivering mechanisms. Given that the capillary-to-myofiber interface is involved in the exchange of substrates and metabolites and the circulation of muscle produced heat, an improved muscle capillary density may enhance skeletal

muscle thermogenesis. Second, local muscle cooling post-exercise has gained recent popularity as an ergogenic aid to improving performance outcomes with exercise. Whilst initial uses of muscle cooling was to reduce soft tissue injury to improve subsequent training session performance, recent evidence has suggested cooling post-exercise to improve the angiogenic response of exercise training (D'Souza et al., 2018). Finally, as stated above, cold stress has been cited as a confounding factor influencing skeletal muscle angio-adaptative responses observed with ambient hypoxia (Banchero et al., 1985), however unlike ambient hypoxia is largely understudied.

With respect skeletal muscle angio-adaptation, many studies have characterized the impact of cold stress by examining changes in muscle capillarization (Bae et al., 2003; Banchero et al., 1985; Bonaterra et al., 2016; Deveci et al., 2002; Deveci and Egginton, 2003; D'Souza et al., 2018; Duchamp et al., 1992; Egginton et al., 2001; Heroux and St. Pierre, 1956; Herpin et al., 2002; Mathieu-Costello et al., 1998; Santocildes et al., 2021; Sillau et al., 1980; Snyder et al., 1992; Suzuki et al., 1997b, 1997a; Wickler, 1981). Between each of these studies there are obvious sources of discrepancy that can influence results such as temperature, duration of exposure, experimental model (human vs. animal/ hibernator vs. non-hibernator), age (juvenile vs. adult), muscle phenotype, and activity level. When categorizing these 17 articles in a similar manner as I did in my hypoxia review (Figure 4), I observed that cold stress led to an improved muscle CD in 88% of articles (Figure 8). However, with respect to C/F ratio, an indicator of true angiogenesis, things are less clear with 53% of articles reporting an increase while 47% reported no change (Figure 8). Interestingly, this suggest that parallels can be drawn between the effect of cold stress and ambient hypoxia with respect to their impact on skeletal muscle capillarization. It appears that like classical dogma associated with ambient hypoxia, cold stress improves muscle CD, yet whether this is due to myofiber atrophy or true muscle angiogenesis can be debated.

At the molecular level, studies have primarily attempted to characterize the influence of cold stress on angio-adaption by measuring the expression of VEGF-A with few studies investigating THBS1 (Al-horani et al., 2019; D'Souza et al., 2018; Ihsan et al., 2014; Joo et al., 2016; Kim et al., 2005; Krapf et al., 2021; Magalhães et al., 2020; Nascimento et al., 2020; Opichka et al., 2019; Shute et al., 2020; Sugasawa et al., 2016; Wagatsuma et al., 2006; Zak et al., 2017; Zieger et al., 2011) (Figure 8). In these studies, cold stress is applied in the form of ambient air

cooling or cold-water immersion, both passively and with an exercise stimulus. With respect to VEGF-A, it is difficult to determine if cold stress leads to an increased expression with only 62.5% of studies reporting a significant increase (Figure 8). Within these studies majority have measured VEGF-A mRNA expression from vastus lateralis biopsies taken after cold-water immersion post-exercise. Thus, like with ambient hypoxia, the effect of cold stress *per se* on VEGF-A expression can be difficult to parse from confounding variables such as the effect of exercise, implication of the nervous system, or the contribution of circulating factors. Moreover, like with ambient hypoxia, the methodological approach of measuring VEGF-A mRNA can be questioned, given that increased mRNA does not necessarily reflect greater biological activity of VEGF-A. At the *in-vitro* level, only two studies have shown cold stress *per se* to influence VEGF-A expression in muscle cells (Krapf et al., 2021; Sugasawa et al., 2016) (Figure 8). In comparison, only a single study performed *in-vitro* has shown THBS1 expression to be influenced by cold stress *per se*, yet these results were observed in human coronary endothelial cells (Zieger et al., 2011) (Figure 8).

FIGURE 8

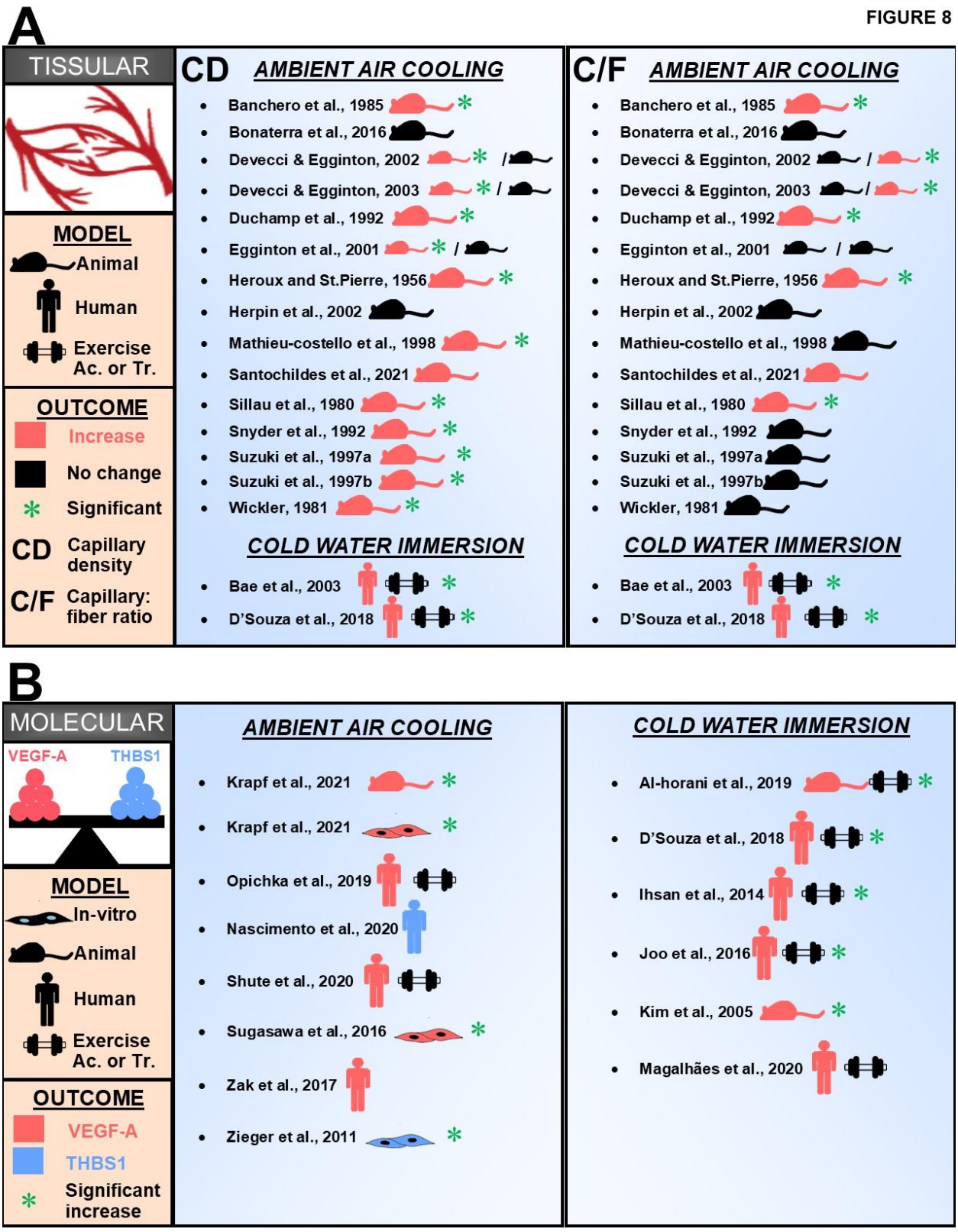

A
TISSULAR
MODEL
Animal
Human
Exercise Ac. or Tr.
OUTCOME
Increase
No change
Significant
CD Capillary density
C/F Capillary: fiber ratio
CD AMBIENT AIR COOLING
Banchero et al., 1985
Bonaterra et al., 2016
Devecci & Egginton, 2002
Devecci & Egginton, 2003
Duchamp et al., 1992
Egginton et al., 2001
Heroux and St.Pierre, 1956
Herpin et al., 2002
Mathieu-costello et al., 1998
Santochildes et al., 2021
Sillau et al., 1980
Snyder et al., 1992
Suzuki et al., 1997a
Suzuki et al., 1997b
Wickler, 1981
COLD WATER IMMERSION
Bae et al., 2003
D'Souza et al., 2018
C/F AMBIENT AIR COOLING
Banchero et al., 1985
Bonaterra et al., 2016
Devecci & Egginton, 2002
Devecci & Egginton, 2003
Duchamp et al., 1992
Egginton et al., 2001
Heroux and St.Pierre, 1956
Herpin et al., 2002
Mathieu-costello et al., 1998
Santochildes et al., 2021
Sillau et al., 1980
Snyder et al., 1992
Suzuki et al., 1997a
Suzuki et al., 1997b
Wickler, 1981
COLD WATER IMMERSION
Bae et al., 2003
D'Souza et al., 2018
B
MOLECULAR
VEGF-A
THBS1
MODEL
In-vitro
Animal
Human
Exercise Ac. or Tr.
OUTCOME
VEGF-A
THBS1
Significant increase
AMBIENT AIR COOLING
Krapf et al., 2021
Krapf et al., 2021
Opichka et al., 2019
Nascimento et al., 2020
Shute et al., 2020
Sugasawa et al., 2016
Zak et al., 2017
Zieger et al., 2011
COLD WATER IMMERSION
Al-horani et al., 2019
D'Souza et al., 2018
Ihsan et al., 2014
Joo et al., 2016
Kim et al., 2005
Magalhães et al., 2020

Figure 8. The effect of cold stress on tissular and molecular aspects of skeletal muscle angio-adaptation.

(A) The effect of cold stress, both ambient air cooling and cold-water immersion, on tissular adaptations of skeletal muscle capillary density (left table) and skeletal muscle capillary to fiber ratio (right table), in 17 original research articles. and represent if animal or human models were used in the study, respectively. represents if exercise was involved in the study both acute and/or training. The colours **RED**, and **BLACK**, represent if there was an increase (>10% increase) or no change (less than a 10% increase), respectively. * represents if the increase was significant. **CD** and **C/F** represent capillary density or capillary to fiber ratio, respectively. **(B)** The effect of cold stress on changes in molecular expression of the pro-angiogenic vascular endothelial growth factor-A (VEGF-A) and anti-angiogenic thrombospondin-1 (THBS1) *in-vitro* and *in-vivo*, in ambient air cooling or cold-water immersion in 13 original research articles. The colours **RED**, and **BLUE**, represent if the study measured VEGF-A or THBS1, respectively. , , and , represent if the model used was *in-vitro* or in animals or humans, respectively. represents if exercise was involved in the study both acute and/or training. * represents a significant change.

Thus, with respect to skeletal muscle angio-adaptation, many similarities can be drawn between ambient hypoxia and cold stress.

(1) Both cold stress and ambient hypoxia lead to an improved muscle CD, however whether this is due to myofiber atrophy or true muscle angiogenesis is debated.

(2) Both cold stress and ambient hypoxia have gained recent popularity as ergogenic aids to improving the angiogenic effect of exercise.

(3) Many studies have sought to describe the effect of cold stress and ambient hypoxia on skeletal muscle angio-adaptation *in-vivo* through measuring VEGF-A mRNA from vastus lateralis biopsy samples.

(4) THBS1 and VEGF-A have been described as key regulators of skeletal muscle angio-adaptation, however THBS1 is largely understudied in the context of both cold stress and ambient hypoxia.

(5) Due to confounding variables in both laboratory and field studies such as nutritional status, physical activity levels, muscle innervation, contribution of circulating factors, duration and intensity of exposure, experimental model used (animal vs human, hibernator vs. non-hibernator) and altered photoperiods, it is difficult to elucidate the contribution of cold stress or ambient hypoxia *per se* on skeletal muscle angio-adaptation.

Therefore, in **Study 2**, for the first time an *in-vitro* approach will be taken to elucidate the impact of cold stress *per se* on angio-adaptive processes. In particular, the effect of *in-vitro* cold stress on myotubes, skeletal muscle endothelial cells, and myofiber-to-endothelial cell communication. **Study 2** serves as the original research component of the thesis.

3.3. Research question.

What is the impact of cold stress *in-vitro* on skeletal muscle angio-adaption?

3.4. Hypothesis.

In-vitro cold stress exerts anti-angiogenic effects on muscle cells and endothelial cells.

Rationale. My hypothesis may sound contrary to what I have been describing in my literature review, that cold stress leads to an improved skeletal muscle capillarization. Yet, these *in-vivo* studies (Figure 8) have various confounding variables such as exercise training, photoperiod, hypoxia, and muscle innervation, to name a few. As a result, it is difficult to demonstrate that cold *per se* stimulated improvements in muscle capillarization. Furthermore, it is important to note that within these studies it is unclear whether the improved skeletal muscle capillarization is a result of myofiber atrophy or true angiogenesis. In comparison, two studies have been performed in-*vitro* to allow for the elucidation of cold stress *per se*. First, Krapf and colleagues (Krapf et al., 2021) have demonstrated that 18h of cold stress culture (18℃) significantly reduces VEGF-A protein secretion by primary human myotubes. Second, Zieger and colleagues (Zieger et al., 2011) have shown 72h of culture in cold stress conditions (25℃) to significantly increased THBS1 protein expression in human coronary endothelial cells. Certainly, myotubes are not necessarily reflective of whole muscle tissue, and endothelial cells from coronary origin are not necessarily reflective of those from skeletal muscle tissue. However, these two limited examples *in-vitro* suggest that cold stress *per se* is anti-angiogenic in myotubes and endothelial cells, by decreasing the secretion of VEGF-A, and stimulating the expression of THBS1, respectively.

3.5. Research aims.

(1) Assess how cold stress exposure influences the expression of angiogenic molecules in differentiated C2C12 myotubes.

(2) Assess if cold stress can indirectly affect the migration of primary skeletal muscle endothelial cells by modulating the secretion of angiogenic myokines from cold-exposed differentiated C2C12 myotubes.

(3) Assess the direct effect of cold stress exposure on primary murine skeletal muscle endothelial cells.

(4) Assess the angio-adaptive effect of cold stress on ex-vivo vastus lateralis biopsy samples from C57BL/6 mice.

3.6. Detailed research aims.

(1) Assess how cold stress exposure influences the expression of angiogenic molecules in differentiated C2C12 myotubes.

a) *Objectives.* The effect of cold stress on the expression of THBS1 and VEGF-A, along with a wider screening of angiogenic molecules, will be assessed in the intra- and extracellular lysates of differentiated C2C12 myotubes. As stated in the above sections, THBS1 and VEGF-A have been identified as key angiogenic molecules, however associating the angio-adaptive milieu to these two molecules is a reductionist audit. Therefore, THBS1 and VEGF-A protein expression will be assessed by immunoblotting and ELISA, respectively, whereas 52 additional angiogenic molecules will be assessed by a mouse angiogenesis proteome profiler array.

b) *Expected results.* Based on previous literature *in-vitro* (Krapf et al., 2021; Zieger et al., 2011), I expect cold stress to lead to an increased expression of THBS1 and a decreased expression of VEGF-A, in both intra- and extracellular lysates.

c) *Limitations to this approach.* Time points selected may limit a full appreciation of the effect of cold stress on the expression of angiogenic molecules based on kinetic differences in molecular expression.

(2) Assess if cold stress can indirectly affect the migration of primary skeletal muscle endothelial cells by modulating the secretion of angiogenic myokines from cold-exposed differentiated C2C12 myotubes.

a) *Objectives.* The indirect effect of cold stress on primary skeletal muscle endothelial cells (SMECs) migration will be assessed by treating SMECs with cold conditioned extracellular lysate from C2C12 myotubes in a Boyden chamber chemotaxis assay.

b) *Expected results.* Treatment of SMECs with cold conditioned C2C12 myotube extracellular lysate will stimulate migration to a lesser extent than that of control conditioned extracellular lysate. I would expect this based on the expression of THBS1 and VEGF-A in C2C12 extracellular lysates (see above, Detailed research aims (1) b).)

c) *Limitations to this approach.* Placing conditioned C2C12 myotube extracellular lysate in the lower chamber of the Boyden chamber may circumvent the direct interaction of SMECs with molecules in the extracellular lysate that are relevant to angio-adaptation.

(3) Assess the direct effect of cold stress exposure on primary murine skeletal muscle endothelial cells.

a) *Objectives.* The direct effect of cold stress on the expression and activation of VEGF-receptor 2 (VEGFR2) in SMECs will be assessed, in addition to SMECs proliferation and migration. As stated in the literature review, VEGFR2 activation promotes endothelial cell proliferation and migration, yet represents an avenue through which THBS1 antagonizes VEGF-A signalling. Thus, basal and phosphorylated VEGFR2 expression will be assessed by immunoblotting in SMECs treated with or without recombinant VEGF-A. SMECs proliferation will be assessed by measuring cellular DNA content through fluorescence emission. SMECs migration will be assessed by a Boyden chamber chemotaxis assay.

b) *Expected results.* Current literature demonstrates cold stress *in-vitro* to attenuate proliferation and migration of various cell type (Fujita, 1999; Fulbert et al., 2019; Zhang et al., 2012). Therefore, I would expect direct cold stress to reduce SMECs proliferation and migration, furthermore, this will coincide with reduced VEGFR2 expression and activation.

c) *Limitations to this approach.* As stated in the literature review, a plethora of molecules affect endothelial cell angiogenic responses, thus assessing VEGFR2 expression alone limits the breadth of this study.

(4) Assess the angio-adaptive effect of cold stress on ex-vivo vastus lateralis biopsy samples from C57BL/6 mice.

a) *Objectives.* Vastus lateralis biopsy samples from C57BL/6 mice will be exposed to cold stress and screened at the intra- and extracellular levels for a wide range of angiogenic molecules. While myocytes make up a large portion of muscle tissue, a plethora of other cell types are also present in whole muscle such as neurons, macrophages, pericytes and tenocytes, to name a few. Thus, a mouse angiogenesis proteome profiler array will be used to assess if cold stress leads to whole muscle producing a different angiogenic microenvironment from that of C2C12 myotubes exposed to cold.

b) *Expected results.* I would expect whole muscle exposed to cold stress to produce a different angiogenic microenvironment from that of C2C12 myotubes exposed to cold stress.

c) *Limitations to this approach*. If differences in proteomic results are observed between whole muscle and C2C12 myotubes exposed to cold, it would not be feasible to delineate the specific cell type(s) in whole muscle responsible.

3.7. Methods.

Cell cultures.

Mouse skeletal muscle myoblasts (C2C12) (ATCC, Cat. CRL-1772, Cedarlanes, Burlington, ON, Canada) were cultured in low glucose (1g/L) Dulbecco's modified eagle medium (DMEM) containing L-glutamine and sodium pyruvate (Cat. 319-005-CL, Wiesent Bioproducts, St Bruno, QC, Canada) and supplemented with 1X penicillin-streptomycin solution (Cat. 450-201-EL, Wiesent Bioproducts, St Bruno, QC, Canada) and 10% Performance fetal bovine serum (FBS) (Cat. 098-150, lot 185717, Wiesent Bioproducts, St Bruno, QC, Canada). Upon reaching about 90-95% cell confluence, myoblast differentiation into myotubes was induced within 7 days by replacing 10% FBS with 2% horse serum (Cat. 065-105, lot 52659, Wiesent Bioproducts, St Bruno, QC, Canada).

Mouse C57BL/6 primary skeletal muscle microvascular endothelial cells (SMECs) (Cell Biologics Inc., Cat. C57-6220, Cedarlanes, Burlington, ON, Canada) were plated on gelatin-coated dishes (1.5% Gelatin Type A (BioShop, Cat. Gel771.500, BioShop Canada Inc, Burlington, ON, Canada)) and cultured in complete endothelial cell medium (Cell Biologics Inc., Cat. M1168, Cedarlanes, Burlington, ON, Canada). Cells were used between passages P4 and P8 at a density of about 350,000 cells/60-mm dish.

Rationale. Differentiated C2C12 myotubes were selected as an *in-vitro* model of myofibers for this study for the follow reasonings. First, differentiated C2C12 myotubes have been shown to be a source of production of the key angiogenic molecules, VEGF-A and THBS1 (Kosmidou et al., 2001; Yadav et al., 2014). Second, Little and Seebacher (Little and Seebacher, 2016) have cultured C2C12 myoblasts and differentiated C2C12 myotubes for 10 and 5 days, respectively, at 32℃, suggesting these cells are robust for prolonged durations of *in-vitro* cold stress. Third, differentiated C2C12 myotubes have a predominantly fast-twitch glyolytic phenotype based on myosin heavy chain expression (Abdelmoez et al., 2020; Meissner et al., 2007). Given that Type IIx fibers have been shown to be the largest source of VEGF-A production (Birot et al., 2003), using differentiated C2C12 myotubes may potentiate the possibility of observing an effect of cold stress on VEGF-A production. Finally, electrostimulation of C2C12 myotubes is a well-established model of *in-vitro* exercise (Evers-van Gogh et al., 2015). Thus, C2C12 myotubes can serve as an *in-vitro* model to investigate the effect of cold stress superimposed with exercise or post-exercise muscle cooling.

Primary murine skeletal muscle endothelial cells were chosen in comparison to other endothelial cell types for two reasons. First, like C2C12, they both are derived from mouse origin. Second, because this study aims to investigate the effect of cold stress on skeletal muscle angio-adaptation *in-vitro*, endothelial cells derived from skeletal muscle origin would be most appropriate.

Cold stress conditioning of skeletal muscle endothelial cells and myotubes.

Differentiated C2C12 myotubes and SMECs were cultured under control (37˚C) or cold stress (28˚C) conditions from 6h up to 48h depending on subsequent experiments. In each independent experiment, the analysis of the impact of cold exposure was always made by comparing differentiated C2C12 myotubes obtained from the same original myoblast batch, or by comparing SMECs from the same original SMEC batch, same passage, and same cell seeding density.

C2C12 conditionings. A first C2C12 cold conditioning was performed, composed of three independent experiments where cells were exposed for 6h and 24h to 37˚C or 28˚C (n=6 cell culture dishes per condition). These samples were used for characterizing the impact of 6h and 24h of cold exposure on intracellular and extracellular THBS1 protein expression levels as well as on THBS1 mRNA levels.

A second C2C12 cold conditioning was performed to further characterize the impact of cold exposure on THBS1 protein expression, particularly by analyzing the impact of successive cooling, re-warming and re-cooling. This conditioning was composed of two independent experiments, each of them with 4 conditions (n=6 cell culture dishes per condition). First condition: 36h exposure at 37˚C (normothermic control condition); second condition: 24h at 37˚C followed by 12h of cold exposure at 28˚C (cold exposure, 36h of cell culture); third condition: 12h at 37˚C followed by 12h of cold exposure (28˚C) followed by 12h of re-warming at 37˚C (re-warming, 36h of cell culture); fourth condition: 12h of cold exposure (28˚C) followed by 12h of re-warming (37˚C) followed by 12h of re-cooling at 28˚C (re-cooling, 36h of cell culture).

A third C2C12 cold conditioning was performed where C2C12 were exposed to 37˚C or 28˚C for 24h (n =6 cell culture dishes per condition). Intracellular and extracellular proteins were isolated to measure THBS1 and VEGF-A protein expression levels, to run a mouse angiogenesis proteome profiler array, and to evaluate the impact of secreted muscle proteins on SMECs migratory activity.

SMEC conditioning. A first SMECs cold conditioning was performed to assess the impact of direct cold exposure on endothelial cell migratory activity. SMECs were exposed to 16h pre-conditioning at 37˚C or 28˚C before 6h cell migration at 37˚C in the Boyden chamber chemotaxis assay. Five independent assays were performed with n=6 migration well per condition. Cell proliferation was analyzed in these SMECs by quantifying proliferation at 24h and 48h at 37˚C and 28˚C (3 independent experiments with n=4-8 samples per condition).

A second SMECs cold conditioning was performed to assess the expression of VEGF receptor-2 (VEGFR2) and its responsiveness to stimulation with murine recombinant VEGF-A_{165} (mVEGF-A165, Peprotech, Cat. 450-32-10UG, lot. 031199 K1621, Cedarlanes, Burlington, ON, Canada). SMECs were pre-conditioned at 37˚C or 28˚C for 23h (12h in complete endothelial cell media and 11h in starved media with 1% FBS only) before being incubated with mVEGF-A165 (100ng/ml) for 1h.

Rationale. As a model of *in-vitro* cold stress, 28℃ was selected based on muscle temperature recorded in response to cold-water immersion. Average ice-bath temperature and duration during cold water immersion for sport performance is 10-15℃ for 5-15mins (Broatch et al., 2018; Versey et al., 2013). At this temperature and exposure, specific limb cooling reduces muscle temperature in brachioradialis and vastus lateralis has been recorded at roughly 27.5℃ and 28℃, respectively (Barcroft and Edholm, 1946; Clarke et al., 1958; Ihsan et al., 2014). Furthermore, these examples of muscle temperature in response to specific limb cooling were used instead of muscle temperature during whole body cooling. This is because during whole body cooling core temperature is challenged (Kiley et al., 1984), and as such may elicit a shivering response (Castellani and Young, 2016), representing a potential confounding variable influencing muscle temperature.

The time points of 6h to 24h were selected based on preliminary data showing 2h of cold stress to have a lack of influence on angiogenic proteins (THBS1) in differentiated C2C12 myotubes. In comparison, at 6h a modest increase in expression was observed and a strong increase at 24h.

Stimulation of SMECs with 100ng/ml of recombinant VEGF-A for 1h was based off previous publications from the lab (Aiken et al., 2016) showing this concentration and duration to induce phosphorylation of targets related to VEGFR2 signalling.

Ex vivo skeletal muscle cold conditioning.

Vastus lateralis muscles were collected from sedentary 11-weeks old C57BL6 female mice (027C57Bl/6, Charles River Laboratory, Senneville, QC, Canada) accordingly to the guidelines of the Canadian Council on Animal Care and York University Committee on Animal Care (Approval #2020-05). These muscles were extra samples from a separate research project that were kindly provided by my second reader, Dr.Roudier, who has Animal Care Committee approval (Approval #2020-05). Both myself and my supervisor, Dr.Birot, have been listed on this separate approved research project. Animals were kept in a 12:12 light:dark cycled, temperature-controlled environment with water and diet ad libitum in order to acclimatize to the animal facility. Mice were fasted for 2 hours before anesthesia with isoflurane. Vastus lateralis muscles were collected, weighted, and divided into two smaller portions of about 40-60 milligrams. Mice were euthanized by heart removal. Ex vivo muscle incubation (EMI) assay was adapted from Aiken et al. (Aiken et al., 2016). Briefly, portions of vastus lateralis muscles were rinsed three times in cold PBS

before incubation for 24h at 37˚C or 28˚C in low glucose (1g/L) DMEM (Cat. 319-005-CL, Wiesent Bioproducts, St Bruno, QC, Canada) with 10% Performance FBS (Cat. 098-150, lot 185717, Wiesent Bioproducts, St Bruno, QC, Canada), 1X penicillin-streptomycin solution (Cat. 450-201-EL, Wiesent Bioproducts, St Bruno, QC, Canada). Following incubation, muscle biopsies were rinsed in cold phosphate buffered saline (PBS), homogenized with a MM400 tissue lyser (30 pulse/sec., 4˚C, Retsch GmbH, Haan, Germany). Following centrifugation (12,000 g, 4˚C, 15 min.) protein lysates were concentrated on 3kDa molecular weight cut-off (MWCO) protein concentrators (Pierce, Cat. 88512, ThermoFisher Scientific, Burlington, ON, Canada), and stored at -80˚C until further analysis.

Rationale. In addition to myocytes, whole muscle tissue contains various cell types such as fibroblasts, pericytes, macrophages, satellite cells, and nerve cells, to name a few. Therefore, exposing whole muscle vastus lateralis biopsies samples to cold stress *ex-vivo* provides a closer representation to whole muscle exposed to cold *in-vivo* than exposing C2C12 myotubes alone to cold stress.

Isolation of intracellular and secreted proteins, and total protein concentration determination.

C2C12 and SMECs intracellular protein extracts were collected after cell lysis in a buffer containing 50 mM Tris-base, 100 mM sodium chloride (NaCl), 5 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium deoxycholate, 1% triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na3VO4), protease inhibitor cocktail (Roche Complete Mini, Cat. 04906845001, Sigma-Aldrich, Oakville, ON, Canada), phosphatase inhibitor cocktail (Roche PhosSTOP, Cat. 11836153001, Sigma-Aldrich, Oakville, ON, Canada), pH 8. After 20 minutes lysis incubation at 4˚C, homogenates were centrifugated at 16,000 g and 4˚C for 15 minutes, and supernatants were collected.

Secreted proteins from C2C12 myotubes were collected from cell medium. Protein extracts were concentrated by loading 100 kDa and 3kDa MWCO protein concentrators (Pierce, Cat. 88503 and 88512, ThermoFisher Scientific, Burlington, ON, Canada) with 500 µl of cell medium. 100 kDa and 3 kDa concentrators were centrifugated at 12,000 g for 10 and 30 minutes, respectively.

Total protein concentration was determined in intracellular and secreted samples by bicinchoninic acid assay (Sigma-Aldrich, Cat. B9643, Oakville, ON, Canada) as previously described (Aiken et al., 2016; Lam et al., 2021; Roudier et al., 2012, 2010).

Immunoblotting of intracellular and secreted proteins.

Immunoblotting for intracellular proteins was carried out on protein extracts from differentiated C2C12 myotubes, SMECs, and ex vivo muscle incubation (EMI) cultured at 37˚C or 28˚C from 6h up to 24h. After protein isolation and determination of total protein concentration, 30 µg of intracellular C2C12 proteins, 100 µg of extracellular C2C12 proteins, 30 µg of intracellular SMECs proteins, or 30 µg of intracellular EMI proteins were diluted in a 1/5 vol./vol. ratio in 2.69 mM sucrose, 0.28 M sodium dodecyl sulfate (SDS), 2.84 M b-mercaptoethanol, 14 µM bromophenol blue, 0.5 M Tris-base pH 6.8, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted onto nitrocellulose membranes (Whatman Cat. BA95; Sigma-Aldrich, Oakville, ON, Canada). Inter-gels comparison was done by using inter-gels lane average comparison. Quality of the transfer was controlled by Ponceau S Red staining. After blocking with 5% fat-free milk in 0.1% tween tris-buffered saline buffer, membranes were probed with the following primary antibodies: mouse monoclonal anti-thrombospondin-1 (1:200, clone A6.1, Invitrogen, Cat. MA513398, ThermoFisher Scientific, Burlington, ON, Canada); rabbit monoclonal anti-thrombospondin-1 (1:1000, clone D7E5F, Cell Signaling, Cat. 37879, New England Biolabs, Whitby, ON, Canada); rabbit monoclonal anti-Phospho-VEGF Receptor-2 (Tyr1175) (1:1000, clone D5B11, Cell Signaling, Cat. 3770, New England Biolabs, Whitby, ON, Canada); rabbit monoclonal anti-VEGF Receptor-2 (1:1000, clone D5B1, Cell Signaling, Cat. 9698, New England Biolabs, Whitby, ON, Canada); rabbit monoclonal anti-RBM3 (1:1000, clone EPR6061(2), Cat. ab134946, Abcam, Toronto, ON, Canada); rabbit monoclonal anti-thrombospondin-4 (1:1000, clone EPR22922-232, Cat. ab263898, Abcam, Toronto, ON, Canada); rabbit polyclonal anti-ATF6 (1:1000, Cat. ab37149, Abcam, Toronto, ON, Canada); rabbit polyclonal anti-Grp78 (1:1000, Cat. ab21685, Abcam, Toronto, ON, Canada). β-actin and α/β-tubulin were detected as protein loading controls for the analysis of intracellular proteins (mouse monoclonal anti-b-actin, 1:1000, clone C4, Cat. sc-47778, Santa-Cruz Biotechnology, Santa-Cruz, CA, USA; rabbit polyclonal anti-a/b-tubulin, 1:1000, Cell Signaling, Cat. 2148, New England Biolabs, Whitby, ON, Canada). For extracellular proteins, the Ponceau S staining was used as a

loading control. Membranes were then incubated with the following secondary horseradish peroxidase (HRP) conjugated antibodies: goat anti-rabbit IgG (1:1000, Cell Signaling, Cat. 7074, New England Biolabs, Whitby, ON, Canada), rabbit anti-mouse IgG (1:1000, Dako, Cat. P0260). Proteins were visualized with enhanced chemiluminescence (Pierce enhanced luminol-based chemiluminescence (ECL) Western Blotting Substrate, Cat. 32106, ThermoFisher Scientific, Burlington, ON, Canada; SuperSignal West Atto Ultimate Sensitivity Chemiluminescent Substrate, Cat. A38554, ThermoFisher Scientific, Burlington, ON, Canada; SuperSignal West Dura Extended Duration Substrate, Cat. 34075, ThermoFisher Scientific, Burlington, ON, Canada) on an imaging station (Kodak 4000MM Pro; Carestream Health, Concord, ON, Canada). Images were analyzed and protein expression levels quantified using NIH Image J imaging software.

Total RNA isolation and real-time qPCR.

Total RNAs were isolated from C2C12 myotubes cell lysate following 6h and 24h exposure to 37˚C or 28˚C (n=3 independent experiments with n=6 samples per condition) using the QIAzol Lysis Reagent (Qiagen, Cat. 79306, Toronto, ON, Canada) as previously described (Lam et al., 2021). RNA concentration was determined by measuring absorbance at 260 and 280 nm. RNA was then reverse-transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Cat. 4387406, ThermoFisher Scientific, Burlington, ON, Canada). Semi-quantitative real-time PCR was performed for each sample in triplicate using TaqMan® Gene Expression assay probes and TaqMan® Universal Master Mix II (Applied Biosystems, Cat. 4440042, ThermoFisher Scientific, Burlington, ON, Canada) for 40 cycles of denaturation at 95˚C for 3 seconds, followed by annealing and extension at 60˚C for 30 seconds. Thrombospondin-1 (*THBS-1*) was measured and the ΔΔCt method was used to express the relative changes. mRNA changes were normalized to *HPRT* gene. Data and expressed as $2^{-\Delta\Delta Ct}$.

VEGF-A protein determination by ELISA.

VEGF-A protein concentration was quantified in intracellular and secreted protein samples from C2C12 myotubes cultured for 24h at 37˚C or 28˚C (n=1 independent experiments with n=6 samples per condition) with a mouse VEGF Quantikine ELISA kit (R&D Systems, Cat. MMV00, Cedarlanes, Burlington, ON, Canada) accordingly to the manufacturer's instructions and using respectively 20 µg and 75 µg of total intracellular and secreted proteins.

Mouse angiogenesis proteome profiler array.

C2C12 myotubes and vastus lateralis biopsies from the ex vivo muscle incubation (EMI) were exposed for 24h to either 37˚C or 28˚C. Intracellular and extracellular proteins were isolated as previously described (n=6 per conditions: Intracellular or secreted, 37˚C versus 28˚C). Samples from each group were pooled and total protein concentrations of each pool were assessed by BCA assay. The assay was run accordingly to the manufacturer's instructions (R&D Systems, Cat. ARY015, Cedarlanes, Burlington, ON, Canada) and as previously described (Roudier et al., 2012). An amount of 250-300 µg and 400 µg of total protein from C2C12 myotubes and EMI, respectively, were incubated with the array nitrocellulose membranes pre-probed with primary antibodies against 53 pro- or anti- angiogenic molecules. Target protein spots were visualized by incubating each membrane with a cocktail of biotinylated secondary antibodies and streptavidin-HRP and using an enhanced chemiluminescence procedure (SuperSignal West Dura Extended Duration Substrate, Cat. 34075, ThermoFisher Scientific, Burlington, ON, Canada). Protein spots were quantified using a Kodak Imaging station 4000MM Pro with Carestream software and analyzed using NIH Image J Software.

Boyden chamber migration assay.

Endothelial cell migratory response was assessed by performing Boyden chamber chemotaxis assays as previously described (Aiken et al., 2016). Following trypsinization, SMECs were loaded in the upper chamber at a density of 500 cells/µl. Cells migrated through an 8-µm polycarbonate membrane filter (NeuroProbe, Inc., Cat. PFB8, Cedarlanes, Burlington, ON, Canada) coated with 50 µg/ml collagen (Gibco, Cat. A10438-01, ThermoFisher Scientific, Burlington, ON, Canada) in 0.02 M acetic acid (Sigma-Aldrich # 320099). In each Boyden chamber experiment, the basal level of SMECs migration was assessed by loading the bottom chamber with 28 µl of DMEM supplemented with 1% FBS. A positive control for migration was incorporated on each assay by loading the bottom chamber with 28 µl of DMEM supplemented with 10% FBS and 5% endothelial cell growth supplement (ECGS 100x, ScienCell, Cat.1052, Cedarlanes, ON, Canada). SMECs migration was then assessed in response to stimulation by C2C12-secreted proteins collected after 24h exposure to 37˚C or 28˚C (indirect C2C12-mediated impact of cold) or by pre-conditioning SMECs for 16h at 37˚C or 28˚C (direct impact of cold on SMEC). For indirect and direct evaluation of cold exposure, 6 and 5 independent Boyden chamber experiments were run,

respectively (n=6 migration wells per condition). After 6 hours of migration time at 37˚C, the nitrocellulose membrane filter was washed with cold PBS, fixed in cold methanol, stained with 10% Giemsa stain diluted in water (Cat. R03055-74, Sigma-Aldrich, Burlington, ON, Canada), and mounted on a glass microscope slide. For each experiment, four individual fields of view per well were counted under 40X magnification, and results were presented as the average of all fields of view.

Skeletal muscle endothelial cell proliferation.

SMECs were seeded on a 96-wells plate at 1,000 cells/well density at day 0 and cultured at 37˚C or 28˚C. Cell proliferation was quantified at days 0, 1 and 2 by using the cyQUANT® NF Cell proliferation Assay kit (Invitrogen, Cat. C35007, ThermoFisher Scientific, Burlington, ON, Canada) accordingly to the manufacturer's instructions. Briefly, the assessment of cell proliferation is based on quantifying the fluorescence emitted at 485 nm by a cell-permeant DNA-binding dye. Two independent cell conditionings were performed n=8 cell culture wells per condition (37˚C versus 28˚C) and per time-point (days 0, 1, and 2).

Statistical analysis.

Statistical analyses were performed using un-paired t-test, 1-way or 2-way ANOVA using Prism9 (GraphPad software), Tukey multiple comparison post-tests for ANOVA analyses. $P<0.05$ was considered to be statistically significant.

3.8. Results.

Cold stress induces an increased expression of RNA-binding motif 3 expression in differentiated C2C12 myotubes.

The cold shock protein, RNA-binding motif 3 (RBM3), was measured as a positive control for cold-stress in differentiated C2C12 myotubes exposed to 6h and 24h of cold stress or control conditions (Figure 9A). No significant change in expression was observed after 6h. However, a strong and significant increased expression was observed following 24h of cold stress (Figure 9A: +75% ($P\leq0.0001$) and +90% ($P\leq0.0001$) in two independent experiments).

Cold stress stimulates a robust increase in thrombospondin-1 expression in differentiated C2C12 myotubes.

Thrombospondin-1 (THBS1) intracellular protein expression showed a modest trend for an increase after 6h of cold stress (Figure 9B: +54% (n.s), +39% (P=0.0249), and +42% (n.s) in three independent experiments). In comparison, 24h of cold stress exposure led to a strong and significant increase in intracellular THBS1 protein expression (Figure 9B: +79% (P=0.0023), +126% ($P\leq$0.0001), and +102% (P=0.0003) in three independent experiments). With regards to THBS1 mRNA expression, the shortest cold exposure duration (6h) had no effect, whereas mRNA levels were significantly increased after 24h (Figure 9C: +72% (P=0.0412), +110% ($P\leq$0.0001), and +28% (P=0.0003) in three independent experiments). I then questioned if the increase in THBS1 intracellular protein was a physiological response or simply the reflect of a deleterious effect of cold on C2C12 myotubes. This was answered by exposing C2C12 myotubes to four different experimental conditions (Figure 9D). Condition #1: 37 Control, C2C12 myotubes were cultured for 36h at 37℃. Condition #2: 28 Control, C2C12 myotubes were cultured for 24h at 37℃ followed by 12h of culture at 28℃. Condition #3: Re-warming, C2C12 myotubes were cultured for 12h at 37℃ followed by 12h of culture at 28℃ followed by 12h of culture at 37℃. Condition #4: Re-cooling, C2C12 myotubes were cultured for 12h at 28℃ followed by 12h of culture at 37℃ followed by 12h of culture at 28℃. Regardless of experimental condition, all cell lysates were collected following 36h of culture (Figure 9D). First, 12h of cold stress exposure led to an increased intracellular THBS1 protein expression (Figure 9D: +34% (P=0.0167) and +52% (P=0.0077) in two independent experiments). This response was completely reversed when cells were re-warmed for an equal amount of time (Figure 9D: -37% (P=0.0006) and -36% (P=0.0047) in two independent experiments). Finally, re-cooling the cells after a period of re-warming resulted in a re-stimulation of THBS1 protein expression (Figure 9D: +43% (P=0.0092) and +27% (n.s) in two independent experiments). Therefore, the increased expression in intracellular THBS1 protein following cold exposure is a reversible and re-inducible response.

Cold stress reduces the expression of thrombospondin-1 protein in differentiated C2C12 myotube extracellular lysate.

With respect to skeletal muscle angio-adaptation, THBS1 protein has been described as a secreted myokine influencing muscle capillarization. Thus, despite the increase in intracellular THBS1

protein observed with cold stress, more relevant to skeletal muscle angio-adaptation is extracellular THBS1 protein expression. Therefore, THBS1 protein expression was assessed in the extracellular lysates of the same differentiated C2C12 myotubes used to describe intracellular THBS1 protein following 24h of cold stress (Figure 9B). When immunoblotting for extracellular THBS1 protein, three bands were observed in comparison to a single band intracellularly (C2C12 Intra. on Figure 10A). Contrary to the previous results obtained on intracellular lysates, 24h of cold stress exposure led to a significant reduction in extracellular THBS1 protein expression overall (Figure 10A: -21%, (P=0.0243) when quantifying all bands), and to an even more pronounced reduction when quantifying specifically the lower band alone (Figure 10A: -50%, P=0.0065). These results were surprising given the robust increase in intracellular THBS1 protein previously observed (Figure 9). Therefore, another anti-THBS1 antibody (clone D7E5F versus A6.1 previously) was used to confirm my observations (Figure 10B). Like with the A6.1 clone (Figure 9), 24h of cold stress exposure led to a significant increase in intracellular THBS1 protein expression when immunoblotting was performed with the D7E5F clone (Figure 10B: +171%, $P\leq0.0001$). To investigate why immunoblots for THBS1 showed a single band in the intracellular lysate but three bands in the extracellular lysate, THB1 protein expression was assessed qualitatively in 5 different conditions (Figure 10C). Condition #1: C2C12 intra., differentiated C2C12 myotube intracellular lysate. Condition #2: C2C12 extra., differentiated C2C12 myotube extracellular lysate. Condition #3: Diff media., basal differentiation media. Condition #4: Horse Serum., horse serum. Condition #5: DMEM., basal DMEM without supplemental growth factors. A general background of THBS1 in the differentiation media used to culture differentiated C2C12 myotubes was observed when using both antibodies (Figure 10C). More specifically, this background seemed to be due to the 2% horse serum used for myotube differentiation since no band was detected in DMEM alone (Figure 10C). Based on these results, differentiation media alone (no cells) was then incubated for 24h at 37˚C or 28˚C (Figure 10D). Cold stress did not alter the general background of THBS1 extracellular protein found in the differentiation media. Furthermore, immunoblotting of differentiation media alone revealed only two distinct THBS1 bands (upper and middle) (Figure 10CD). This is of particular importance. Indeed, this suggests that the lower band is likely a contribution exclusively arising from C2C12 secretion. More relevant, this lower band was the only one to be significantly reduced by cold stress exposure. In conclusion, despite a robust

increase in C2C12 intracellular THBS1 expression, cold stress significantly reduced the amount of THBS1 protein in the extracellular lysate.

Differential intra- and extracellular protein expression induced by cold stress is not exclusive to thrombospondin-1.

THBS1 is a member to a larger family of four additional thrombospondin proteins. To investigate if the differential intra- and extracellular protein expression of THBS1 was exclusive to THBS1, the effect of cold stress on intra- and extracellular THBS4 protein expression in the same C2C12 cells used for Figure 9 and Figure 10 was assessed. THBS4 was selected based on its membership as a thrombospondin family member, and a larger size and molecular weight relative to THBS1. Like THBS1, 24h of cold stress led to a significant increase in intracellular THBS4 protein expression (Figure 11A: respectively +61% (P=0.0068), +116% (P≤0.0001), and +78% (P=0.0118) in three independent experiments). This increased expression also showed a trend to be reversible with re-warming (Figure 11B: -20% (n.s) and -23% (n.s) in two independent experiments) and re-inducible with re-cooling (Figure 11B: +29% (n.s) and +28% (n.s) in two independent experiments). When investigating THBS4 protein in the extracellular lysate, 24h of cold stress significantly reduced total THBS4 protein (Figure 11C: -14%, P=0.0068). Furthermore, THBS4 protein was unchanged in differentiation media alone (no cells) that was incubated for 24h in either cold stress or control conditions (Figure 11D). Together these results indicate that the cold stress induced differential intra- and extracellular pattern of expression for THBS1 are also observed with THBS4 and are therefore not THBS1 specific. Based on these observations (increased intracellular and decreased extracellular, THBS1 and -4 protein), I hypothesized that THBS1 and -4 could be serving an intracellular function. Cold stress has been shown to stimulate the upregulation of endoplasmic reticulum-stress (ER-stress) related protein, glucose regulated protein 78 (Grp78), in skeletal muscle (Storey and Storey, 2019). In cardiac muscle, both THBS1 and -4 have been shown to promote the increased expression of ER-stress related proteins, such as Grp78, by facilitating the shuttling of activating transcription factor 6 (ATF6) from the endoplasmic reticulum to the golgi and nucleus. (Brody et al., 2016; Doroudgar and Glembotski, 2013; Lynch et al., 2012). To assess if the cold stress induced increase in intracellular THBS1 and -4 expression was to facilitate an ER-stress response, intracellular ATF6 and Grp78 were immunoblotted (Supplemental Figure 1). Cold stress, for 6h and 24h, did not alter differentiated

C2C12 myotube intracellular protein expression of either ATF6 (Supplemental Figure 1A) or Grp78 (Supplemental Figure 1B).

Cold stress exposure leads to a reduced expression of vascular endothelial growth factor-A in extracellular lysate of differentiated C2C12 myotubes.

THBS1 and VEGF-A have both respectively been described as key anti- and pro- angiogenic molecules for skeletal muscle angio-adaptation (Breen et al., 2008; Lemieux and Birot, 2021; Olfert and Birot, 2011). To further elucidate whether cold stress alters myokine expression to favour a pro- or anti- angiogenic response, the expression of VEGF-A protein was characterized in the intra- and extracellular lysates of differentiated C2C12 myotubes. Cold stress had no effect on intracellular VEGF-A protein (Figure 12A). However, cold stress significantly reduced VEGF-A protein expression in the extracellular lysate (Figure 12B: -37%, P=0.0001). I also confirmed in these samples that cold stress altered THBS1 intra- and extracellular protein expression as previously described in Figure 9 and Figure 10 (Supplemental Figure 2). Cold stress both significantly increased and decreased, intra- (Supplemental Figure 2A: +123% ($P\leq$0.0001) and extracellular (Supplemental Figure 2B: -20% (P=0.0524; n.s) in all bands and -51% (P=0.0019) in the lower band) THBS1 protein expression, respectively.

Screening for 53 angiogenic molecules in differentiated C2C12 myotubes is inconclusive.

Whether cold stress leads to a pro- or anti- angiogenic microenvironment in the extracellular lysate of differentiated C2C12 myotubes could not be answered based on THBS1 and VEGF-A results. Thus, in these same samples (Figure 12 and Supplemental Figure 2), a mouse angiogenesis proteome profiler array was used to screen for 53 angiogenic proteins in both intra- and extracellular lysates (Figure 13A). Of a possible 53 proteins, a total of 22 and 28 proteins were identified in the intra- and extracellular lysate, respectively (Figure 13B, Table 1). Of these proteins, 17 were found to be expressed both in intra- and extracellular lysates (Figure 13B, Table 1). When categorizing these 17 proteins into pro- and anti- angiogenic categories, cold stress led to a mixed intracellular expression of pro-angiogenic molecules with 23% increasing, 31% decreasing, and 46% showing no change (Figure 13C). In comparison, cold stress led to an increased intracellular expression of all anti-angiogenic molecules (Figure 13C). However, in the extracellular lysate, cold stress decreased the expression of all proteins regardless of if pro- or anti-angiogenic (Table 1, Figure 13C). Thus, screening of 53 angiogenic proteins provided no clear

consensus regarding if cold stress induces differentiated C2C12 myotubes myokine expression towards a pro- or anti- angiogenic microenvironment.

The extracellular lysate from cold-conditioned differentiated C2C12 myotubes does not stimulate nor attenuate skeletal muscle endothelial cell migration.

Proteomic results provided no consensus whether the effect of cold stress on C2C12 secreted proteins would lead to a pro- or anti- angiogenic microenvironment. Thus, a Boyden chamber chemotaxis assay was performed to determine if cold-conditioned C2C12 extracellular lysates could preferentially stimulate skeletal muscle endothelial cell (SMECs) migration. A total of six independent Boyden chamber experiments were performed, each with their own internal positive control of cell migration (Figure 14A). In each independent experiment, my positive control stimulation (10% FBS, 5% ECGS) of SMECs significantly increased cell migration relative to non-stimulatory conditions (1% FBS, no ECGS) (Figure 14A: +93% ($P \leq 0.0001$), +92% ($P \leq 0.0001$), +116% ($P = 0.0002$), +60% ($P \leq 0.0001$), +83% ($P \leq 0.0001$), and +122% ($P \leq 0.0001$) in six independent experiments). This validated each assay. The stimulation of SMECs with cold-conditioned extracellular lysate brought no clear consensus (Figure 14B). Cold conditioned extracellular lysate randomly increased or decreased cell migration relatively to SMEC stimulated with warm-conditioned extracellular lysates (Figure 14B).

Direct exposure to cold stress increases the migratory responsiveness of skeletal muscle endothelial cells.

Since my previous results from C2C12 exposure to cold stress provided no consensus regarding if cold stress is pro- or anti- angiogenic on SMECs, I decided to evaluate the impact of a direct exposure of SMECs to cold stress. Here, SMECs were preconditioned to cold stress or control conditions for 16h prior to a Boyden chamber chemotaxis assay (Figure 15). In all five experiments performed, regardless of thermal preconditioning SMECs migratory activity was increased with stimulation (10% FBS, 5% ECGS) relative to non-stimulation conditions (1% FBS, no ECGS) (Figure 15A: 37 C, +76% ($P \leq 0.0001$), +195% ($P \leq 0.0001$), + 159% ($P \leq 0.0001$), +97% ($P \leq 0.0001$), +103% ($P \leq 0.0001$); 28 C, +105% ($P \leq 0.0001$), +341% ($P \leq 0.0001$), +251% ($P \leq 0.0001$), +185% ($P \leq 0.0001$), +129% ($P \leq 0.0001$), in five independent experiments). More importantly, when assessing SMECs stimulated migration as a fold-increase relative to respective non-stimulatory conditions (37 Stim/Non-stim vs. 28 Stim/Non-stim), a significant effect of cold

stress to stimulate SMECs migration is observed (Figure 15B: +30% (P=0.007), +146% (P≤0.0001), +92% (P=0.0003), +88% (P=0.0002), +26% (P=0.0548) (n.s), in five independent experiments). Given that the cells are counted at the cessation of the Boyden chamber assay, such count may be influenced by cell migration and/or cell proliferation. SMECs were therefore cultured in cold stress or control conditions for up to two days and assessed for proliferation. SMECs cultured in cold stress conditions showed a drastic attenuation in proliferation (Figure 15C). In comparison, cells cultured in control conditions maintained their proliferation (Figure 15C). Based on these observations, it can be assumed that the greater migratory responsiveness of cold-preconditioned SMECs (Figure 15B) is independent of cell proliferation.

Cold exposure increases skeletal muscle endothelial cells expression of VEGF receptor-2.

VEGF receptor 2 (VEGFR2) is the cognate receptor primarily responsible for facilitating the VEGF-A downstream signalling that promotes endothelial cell proliferation and migration (Wang et al., 2020). VEGFR2 protein was assessed by immunoblotting (Figure 16A). Three distinct bands were observed. Previous reported observations have identified these three forms of VEGFR2 as follows: a 230 kDa fully glycosylated and mature form; a 200 kDa glycosylated intermediate form; and a 150 kDa non-glycosylated form (Takahashi et al., 2001; Takahashi and Shibuya, 1997). Amongst these three forms, only the 230 kDa form is expressed on the cell membrane surface and can be phosphorylated following stimulation with VEGF-A (Takahasi and Shibuya, 1997). In my study, cold stress exposure significantly increased VEGFR2 protein expression, regardless of stimulation with recombinant VEGF-A (mVEGFA165). The effect was particularly significant for the 230k Da and 200 kDa forms (Figure 16A: 230 kDa, + 55% (P=0.0230) and +124% (P=0.0134); 200 kDa, +157% (P=0.0018) and +129% (P=0.0004), in -mVEGF-A165 and +mVEGF-A165 conditions, respectively). The cold-induced increase in the 150 kDa form was significant only in the context of stimulation with recombinant VEGF-A (Figure 16A). Interestingly, the expression of the 230 kDa form was significantly decreased with recombinant VEGF-A stimulation regardless of the temperature (Figure 16A). Such a reduction in the 230 kDa form could reflect the internalization and degradation of VEGFR2, an important process in regulating its signal transduction (Ewan et al., 2006; Lampugnani et al., 2006). Next, VEGFR2 responsiveness to mVEGF-A165 stimulation was assessed by measuring the phosphorylation of the receptor on residue tyrosine 1175 (p-Y1175-VEGFR2) (Figure 16B). Among the numerous phosphorylation

sites described on VEGFR2, Y1175 is critical for VEGFR2 activation and downstream signalling involved in cell proliferation, migration, and survival (Takahashi et al., 2001; Wang et al., 2020). When assessing VEGFR2 activation as a ratio of p-Y1175-VEGFR2 / 230 kDa VEGFR2, a significantly reduced responsiveness to mVEGF-A165 stimulation is observed in SMECs pre-conditioned to cold stress relative to control cells (Figure 16B: -70%, *P*=0.0085). However, this is not surprising given that cold stress led to a significant increase in the total expression level of the 230 kDa form (Figure 16A). When assessing p-Y1175-VEGFR2 expression relative to a loading control, stimulation of SMECs with recombinant mVEGF-A165 significantly increased the expression of p-Y1175-VEGFR2 protein in cell maintained in control conditions (Figure 16B: +108%, *P*=0.0367), whereas a trend for increase was observed in cells maintained in cold stress (Figure 16B: +146%, *P*=0.0537, n.s). Like with C2C12 myotubes (Figure 9A), RBM3 was used as a positive control of cold stress in SMECs cultured in cold stress or control conditions. Cold stress induced a significant increase in RBM3 in SMECs cultured for 24h (Supplemental Figure 3A: +23% (*P*=0.0041) and +14% (*P*=0.0303) in two independent experiments).

Proteomic screening of skeletal muscle biopsies differs from differentiated C2C12 myotubes.

To gain more insight to the angiogenic impact of cold stress exposure on the skeletal muscle tissue, an angiogenesis proteome profiler array was performed on mouse vastus lateralis biopsies incubated *in-vitro* at 37˚C or 28˚C for 24h (ex vivo muscle incubation (EMI) assay). A total of 52 out of the 53 proteins present in the assay were detected both in intracellular and extracellular lysates (Figure 17AB, Table 2). At the intracellular level, a mixed distribution was observed with 30% of proteins showing an increase or no change, and 70% showing a decrease (Figure 17B). A mixed distribution was even more obvious at the extracellular level with 23% of proteins increasing, 25% decreasing, and 52% showing no change (Figure 17B). This contrasts with the results of the proteome array previously reported in C2C12 myotubes where 100% of secreted proteins were decreased (Figure 13B, Table 1). This difference between C2C12 and skeletal muscle tissue protein expression pattern was confirmed when comparing the distribution of the same 28 extracellular proteins detected in both C2C12 and EMI samples (Figure 17C). To ensure the effect of my cold stress conditioning in the vastus lateralis EMI model, intracellular protein levels for RBM3 was qualitatively assessed (Supplemental Figure 4A). Finally, THBS-1 is not present on the proteome array. Quantification of intracellular THBS1 protein expression in EMI

samples by immunoblotting showed an 100% (P=0.029) increase in cold-conditioned muscle biopsies (Supplemental Figure 4B).

FIGURE 9

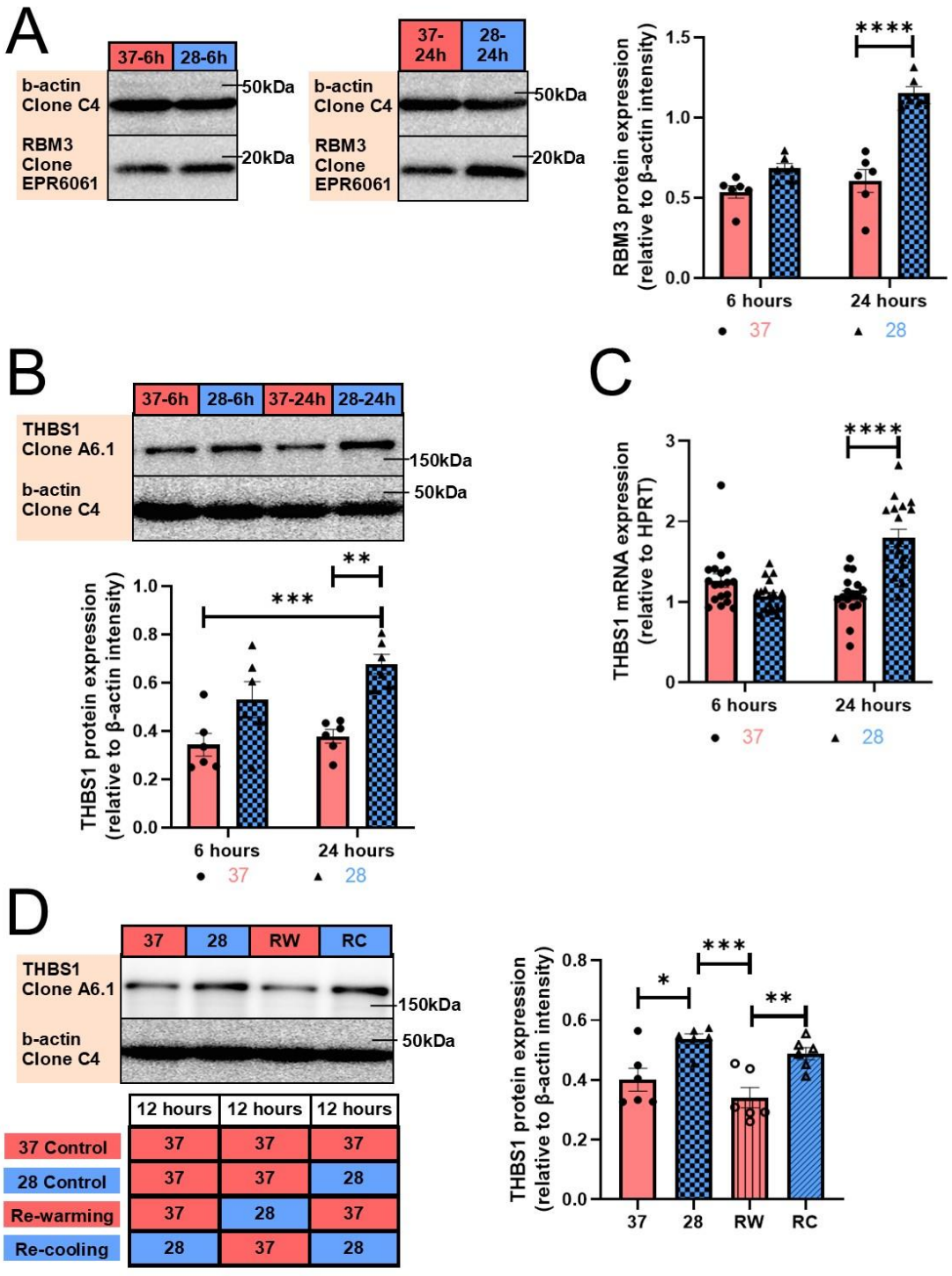

A
37-6h
28-6h
b-actin Clone C4
50kDa
RBM3 Clone EPR6061
20kDa
37-24h
28-24h
RBM3 protein expression (relative to β-actin intensity)
****
6 hours
24 hours
37
28
B
37-6h
28-6h
37-24h
28-24h
THBS1 Clone A6.1
150kDa
THBS1 protein expression (relative to β-actin intensity)
**
***
C
THBS1 mRNA expression (relative to HPRT)
D
37
28
RW
RC
12 hours
12 hours
12 hours
37 Control
28 Control
Re-warming
Re-cooling
*
**

Figure 9. Cold stress increases intracellular RNA-binding motif 3 protein and thrombospondin-1 protein and mRNA expression in differentiated C2C12 myotubes.

(A) Representative immunoblots and densitometry analyses for intracellular cold-shock RNA-binding motif 3 (RBM3) protein expression in differentiated C2C12 myotubes exposed to 6h and 24h of control (37) or cold stress (28) conditions. β-actin was used as a loading control. Data shown is a single representative experiment of two independently conducted experiments with n=6 samples per condition of exposure time and temperature (represented as means ± standard error of the mean (SEM)). Significant difference between groups: ****, $P \leq 0.0001$. **(B)** Representative immunoblots and densitometry analyses for intracellular thrombospondin-1 (THBS1) protein expression in differentiated C2C12 myotubes exposed to 6h and 24h of control (37) or cold stress (28) conditions. β-actin was used as a loading control. Data shown is a single representative experiment of three independently conducted experiments with n=6 samples per condition of exposure time and temperature (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$; ***, $P \leq 0.001$. **(C)** *THBS1* gene expression in differentiated C2C12 myotubes exposed to 6h and 24h of control (37) or cold stress (28) conditions. Hypoxanthinephosphoribosyltransferase-1 (*HPRT*) is used as a housekeeping gene. Data shown is a combined representation of three independently conducted experiments each with n=6 samples per condition of exposure time and temperature (represented as means ± SEM). Significant difference between groups: ****, $P \leq 0.0001$. **(D)** Representative immunoblots and densitometry analyses for intracellular THBS1 protein expression in differentiated C2C12 myotubes exposed to four independent experimental conditions: (1) 37 Control: 36h of culture at 37˚C; (2) 28 Control: 24h of culture at 37˚C followed by 12h of culture at 28˚C; (3) Re-warming:12h of culture at 37˚C followed by 12h of culture at 28˚C followed by 12h of culture at 37˚C; (4) Re-cooling:12h of culture at 28˚C followed by 12h of culture at 37˚C followed by 12h of culture at 28˚C. All cell lysates were collected following 36h of culture regardless of experimental condition. β-actin was used as a loading control. Data shown is a single representative experiment of two independently conducted experiments with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

FIGURE 10

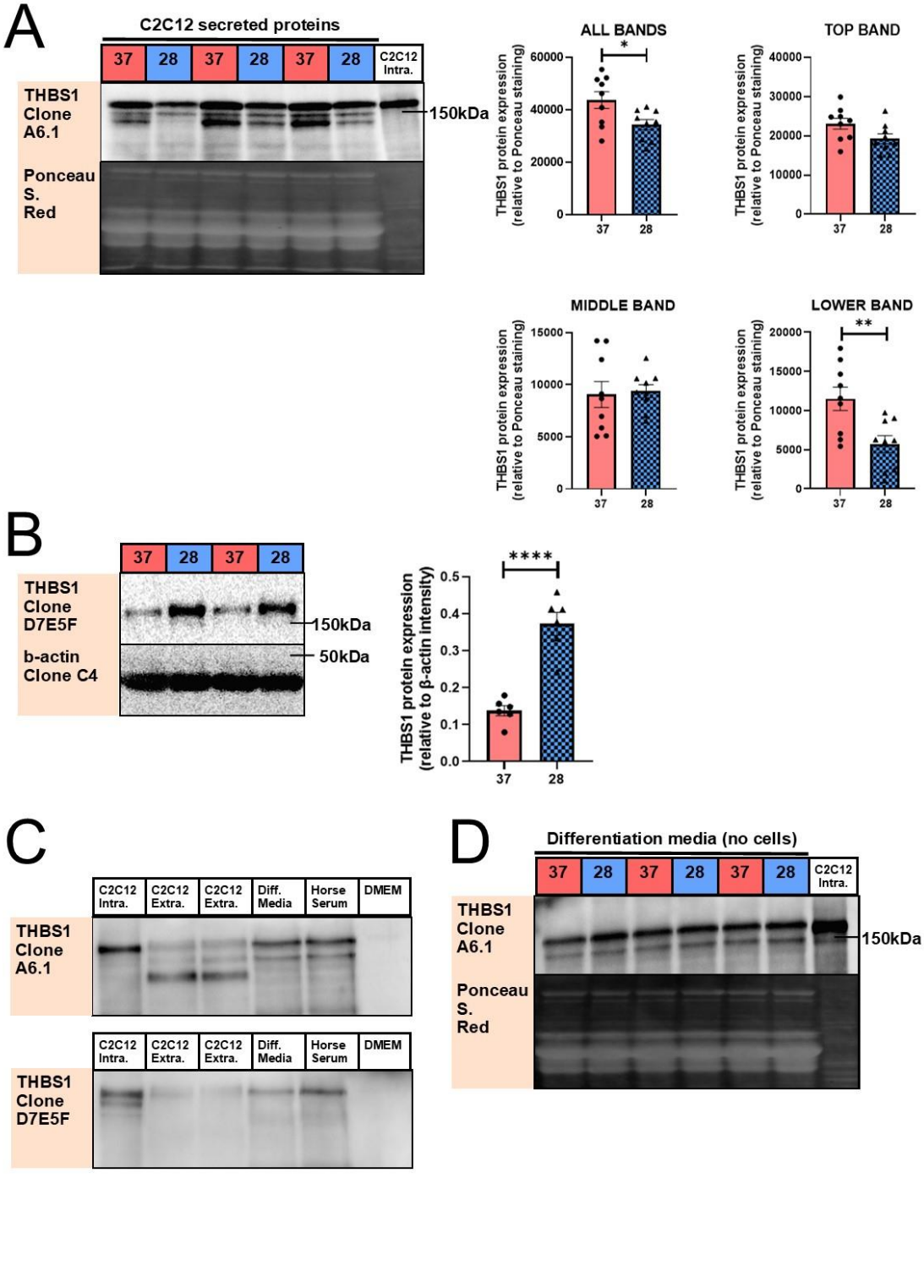

A
C2C12 secreted proteins
37
28
37
28
37
28
C2C12 Intra.
THBS1 Clone A6.1
150kDa
Ponceau S. Red
ALL BANDS
TOP BAND
MIDDLE BAND
LOWER BAND
THBS1 protein expression (relative to Ponceau staining)
B
THBS1 Clone D7E5F
b-actin Clone C4
150kDa
50kDa
THBS1 protein expression (relative to β-actin intensity)
C
C2C12 Intra.
C2C12 Extra.
C2C12 Extra.
Diff. Media
Horse Serum
DMEM
THBS1 Clone A6.1
THBS1 Clone D7E5F
D
Differentiation media (no cells)
C2C12 Intra.
THBS1 Clone A6.1
150kDa
Ponceau S. Red

Figure 10. Extracellular thrombospondin-1 protein expression in differentiated C2C12 myotubes.

(A) Representative immunoblots and densitometry analyses for thrombospondin-1 (THBS1) protein expression in extracellular lysate from differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. Ponceau S Red staining was used to ensure equal protein loading. C2C12 intracellular lysate (C2C12 Intra.) was loaded to visualize discrepancy between band patterns for intra- and extracellular THBS1. Data shown is a combined representation of three independently conducted experiments with n=3 samples per condition (represented as means ± SEM). Significant difference between groups: *, $P \leq 0.05$; **, $P \leq 0.01$. **(B)** Representative immunoblots and densitometry analyses for THBS1 protein expression in differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. Immunoblotting was carried out with D7E5F clone. β-actin was used as a loading control. Data shown is from a single conducted experiment with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: ****, $P \leq 0.0001$. **(C)** Immunoblots of THBS1 protein expression in: differentiated C2C12 myotube intracellular lysate (C2C12 Intra.); differentiated C2C12 myotube extracellular lysate (C2C12 Extra.); differentiation media alone (no cells) (Diff. Media); horse serum alone (no cells) (Horse Serum); and DMEM alone (no cells). THBS1 protein expression was identified using the clone A6.1 in the top panel, and clone D7E5F in bottom panel. **(D)** Representative immunoblots of THBS1 protein expression in differentiation media alone (no cells) that was incubated for 24h in either control (37) or cold stress (28) conditions. Ponceau S Red stain was used to ensure equal protein loading.

FIGURE 11

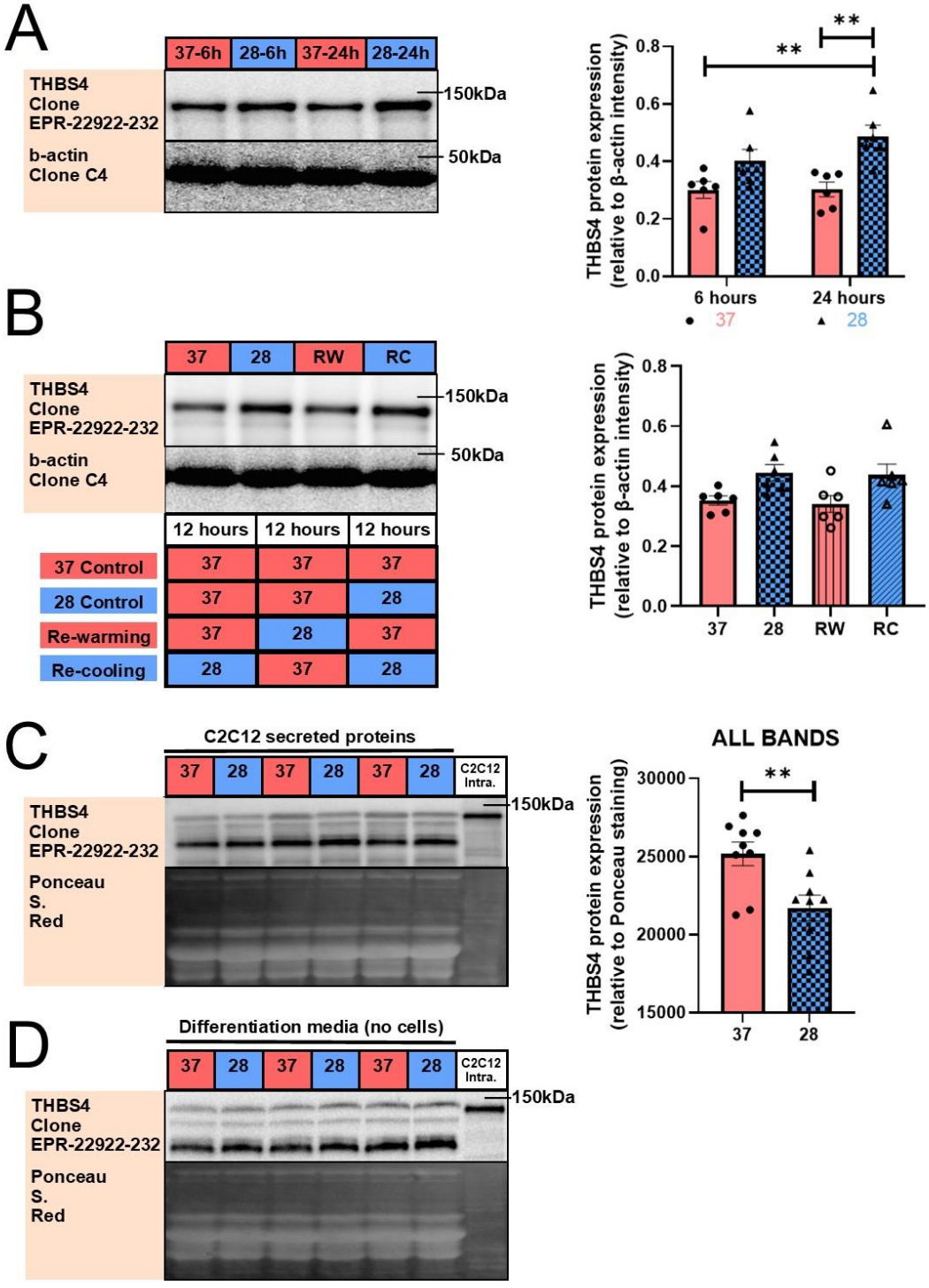

A
37-6h
28-6h
37-24h
28-24h
THBS4
Clone
EPR-22922-232
b-actin
Clone C4
150kDa
50kDa
THBS4 protein expression
(relative to β-actin intensity)
**
**
0.8
0.6
0.4
0.2
0.0
6 hours
24 hours
37
28
B
37
28
RW
RC
THBS4
Clone
EPR-22922-232
b-actin
Clone C4
150kDa
50kDa
12 hours
12 hours
12 hours
37 Control
28 Control
Re-warming
Re-cooling
37 37 37
37 37 28
37 28 37
28 37 28
THBS4 protein expression
(relative to β-actin intensity)
0.8
0.6
0.4
0.2
0.0
37
28
RW
RC
C
C2C12 secreted proteins
37
28
37
28
37
28
C2C12
Intra.
150kDa
THBS4
Clone
EPR-22922-232
Ponceau
S.
Red
ALL BANDS
**
THBS4 protein expression
(relative to Ponceau staining)
30000
25000
20000
15000
37
28
D
Differentiation media (no cells)
37
28
37
28
37
28
C2C12
Intra.
150kDa
THBS4
Clone
EPR-22922-232
Ponceau
S.
Red

Figure 11. Effect of cold stress on thrombospondin-4 protein expression in intra- and extracellular lysates from differentiated C2C12 myotubes.

(A) Representative immunoblots and densitometry analyses for intracellular thrombospondin-4 (THBS4) protein expression in differentiated C2C12 myotubes exposed to 6h and 24h of control (37) or cold stress (28) conditions. β-actin was used as a loading control. Data shown is a single representative experiment of three independently conducted experiments with n=6 samples per condition of exposure time and temperature (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$. **(B)** Representative immunoblot and densitometry analyses for intracellular THBS4 protein expression in differentiated C2C12 myotubes exposed to four independent experimental conditions: (1) 37C Control, 36h of culture at 37˚C; (2) 28C Control, 24h of culture at 37˚C followed by 12h of culture at 28˚C; (3) Re-warming (RW), 12h of culture at 37˚C followed by 12h of culture at 28˚C followed by 12h of culture at 37˚C; (4) Re-cooling (RC), 12h of culture at 28˚C followed by 12h of culture at 37˚C followed by 12h of culture at 28˚C. Cells were collected at the same time, following 36h of culture regardless of experimental condition. β-actin was used as a loading control. Data shown is a single representative experiment of two independently conducted experiments with n=6 samples per condition (represented as means ± SEM). **(C)** Representative immunoblots and densitometry analyses for THBS4 protein expression in extracellular lysate from differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. Ponceau S Red staining was used to ensure equal protein loading. C2C12 intracellular lysate (C2C12 Intra.) was loaded to visualize discrepancy between banding pattern for intra- and extracellular THBS4. Data shown is a combined representation of three independently conducted experiments with n=3 samples per condition (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$. **(D)** Representative immunoblots of THBS4 protein expression in differentiation media alone (no cells) that was incubated for 24h in either control (37) or cold stress (28) conditions. Ponceau S Red stain was used to ensure equal protein loading.

FIGURE 12

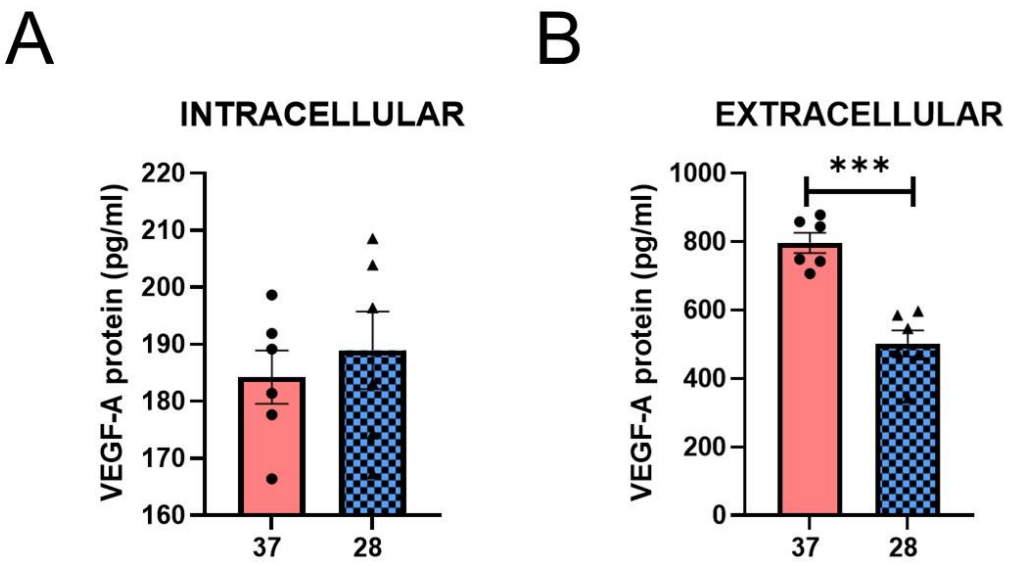

A
INTRACELLULAR
VEGF-A protein (pg/ml)
220
210
200
190
180
170
160
37
28
B
EXTRACELLULAR
***
VEGF-A protein (pg/ml)
1000
800
600
400
200
0
37
28

Figure 12. Effect of cold stress on vascular endothelial growth factor-A protein expression in intra- and extracellular lysates from differentiated C2C12 myotubes.

(A) Representative histogram of intracellular vascular endothelial growth factor-A (VEGF-A) protein expression in differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. Protein expression was quantified by enzyme linked immunodetection sandwich assay (ELISA). Data shown is from a single conducted experiment with n=6 samples per condition (represented as means ± SEM). **(B)** Representative histogram of VEGF-A protein expression in extracellular lysate from differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. Protein expression was quantified by ELISA. Data shown is from a single conducted experiment with n=6 samples per condition (represented as means ± SEM). Significant difference: ***, $P \leq 0.001$.

FIGURE 13

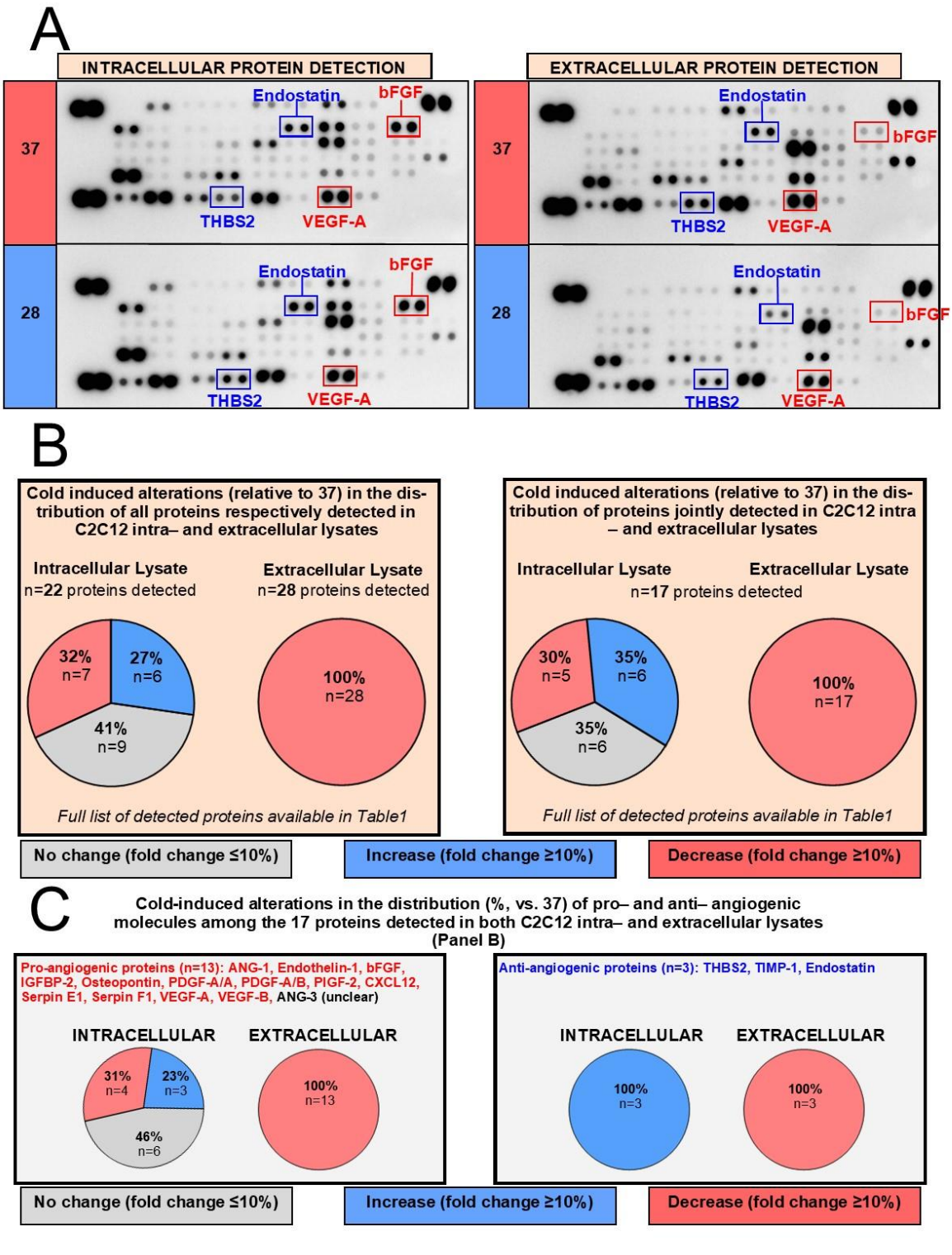
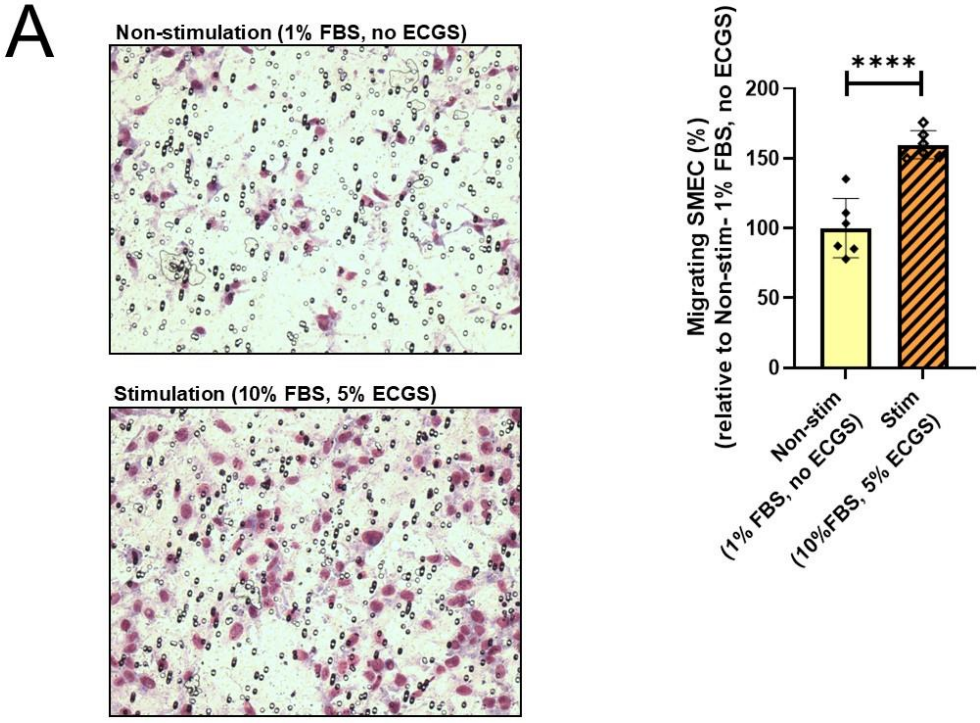

A
INTRACELLULAR PROTEIN DETECTION
EXTRACELLULAR PROTEIN DETECTION
37
28
Endostatin
bFGF
THBS2
VEGF-A
B
Cold induced alterations (relative to 37) in the distribution of all proteins respectively detected in C2C12 intra– and extracellular lysates
Intracellular Lysate
n=22 proteins detected
Extracellular Lysate
n=28 proteins detected
32%
n=7
27%
n=6
41%
n=9
100%
n=28
Full list of detected proteins available in Table1
Cold induced alterations (relative to 37) in the distribution of proteins jointly detected in C2C12 intra – and extracellular lysates
Intracellular Lysate
Extracellular Lysate
n=17 proteins detected
30%
n=5
35%
n=6
35%
n=6
100%
n=17
Full list of detected proteins available in Table1
No change (fold change ≤10%)
Increase (fold change ≥10%)
Decrease (fold change ≥10%)
C
Cold-induced alterations in the distribution (%, vs. 37) of pro– and anti– angiogenic molecules among the 17 proteins detected in both C2C12 intra– and extracellular lysates (Panel B)
Pro-angiogenic proteins (n=13): ANG-1, Endothelin-1, bFGF, IGFBP-2, Osteopontin, PDGF-A/A, PDGF-A/B, PIGF-2, CXCL12, Serpin E1, Serpin F1, VEGF-A, VEGF-B, ANG-3 (unclear)
INTRACELLULAR
EXTRACELLULAR
31%
n=4
23%
n=3
46%
n=6
100%
n=13
Anti-angiogenic proteins (n=3): THBS2, TIMP-1, Endostatin
INTRACELLULAR
EXTRACELLULAR
100%
n=3
100%
n=3
No change (fold change ≤10%)
Increase (fold change ≥10%)
Decrease (fold change ≥10%)

Figure 13. Effect of cold stress on 53 angiogenic molecules in intra- and extracellular lysates from differentiated C2C12 myotubes.

(A) Representative proteome profiler immunoblotting membranes for intra- and extracellular lysates from differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. The anti-angiogenic endostatin and thrombospondin-2 (THBS2) and the pro-angiogenic vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF) are highlighted. Each highlighted square shows duplicate spots for a given protein. **(B)** Distribution in percentage of cold-induced alterations in proteins detected in the proteome profiler array in intracellular and extracellular C2C12 lysates. **(C)** Specific distribution in percentage of cold-induced alterations in pro- and anti- angiogenic proteins detected in the proteome profiler array in intracellular and extracellular C2C12 samples.

FIGURE 14


A
Non-stimulation (1% FBS, no ECGS)
Stimulation (10% FBS, 5% ECGS)
Migrating SMEC (%)
(relative to Non-stim- 1% FBS, no ECGS)
****
200
150
100
50
0
Non-stim
(1% FBS, no ECGS)
Stim
(10%FBS, 5% ECGS)

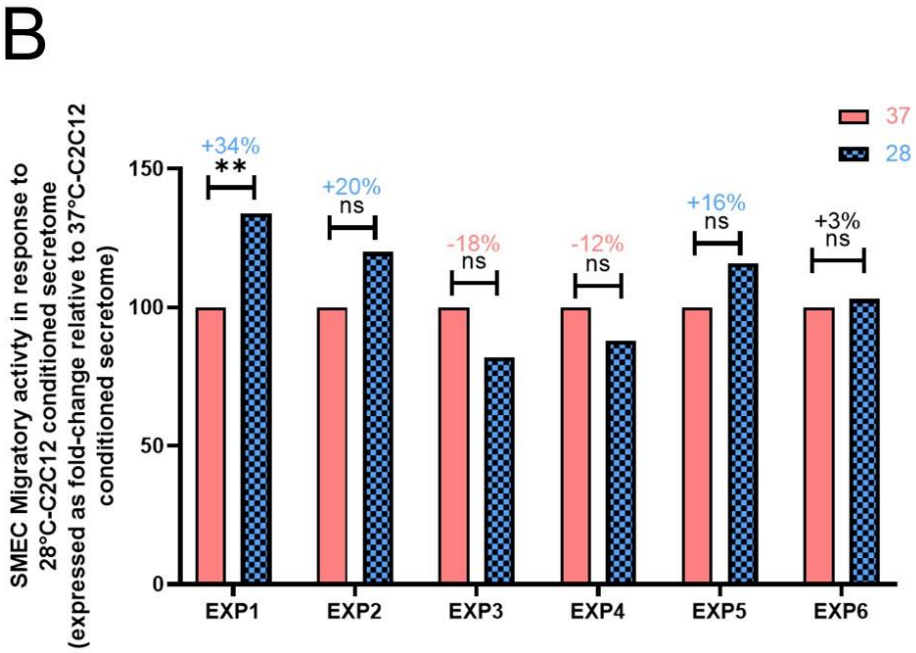

B
SMEC Migratory activty in response to
28°C-C2C12 conditioned secretome
(expressed as fold-change relative to 37°C-C2C12
conditioned secretome)
37
28
150
100
50
0
+34%
**
+20%
ns
-18%
ns
-12%
ns
+16%
ns
+3%
ns
EXP1
EXP2
EXP3
EXP4
EXP5
EXP6

Figure 14. Skeletal muscle endothelial cell migratory activity in response to extracellular lysate from cold stress conditioned differentiated C2C12 myotubes.

(A) Representative images and histogram for skeletal muscle endothelial cell (SMECs) migration in Boyden chamber chemotaxis assay in response to no stimulation (1% FBS, no ECGS) or stimulation (10% FBS, 5% ECGS) for 6h. Migration is expressed as a percentage (%) of migrating cells in stimulatory conditions relative to non stimulatory conditions. Data presented is a single experiment representative of six independent experiments conducted each with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: ****, $P \leq 0.0001$. **(B)** Graphical representation comparing SMECs migratory activity in six independent Boyden chamber chemotaxis experiments in response stimulation with extracellular lysate from control (37) or cold stress (28) conditioned differentiated C2C12 myotubes. Data is represented as means ± SEM with n=6 samples per condition. Each experiment is compared statistically by un-paired t-test. Significant difference between groups: **, $P \leq 0.01$.

FIGURE 15

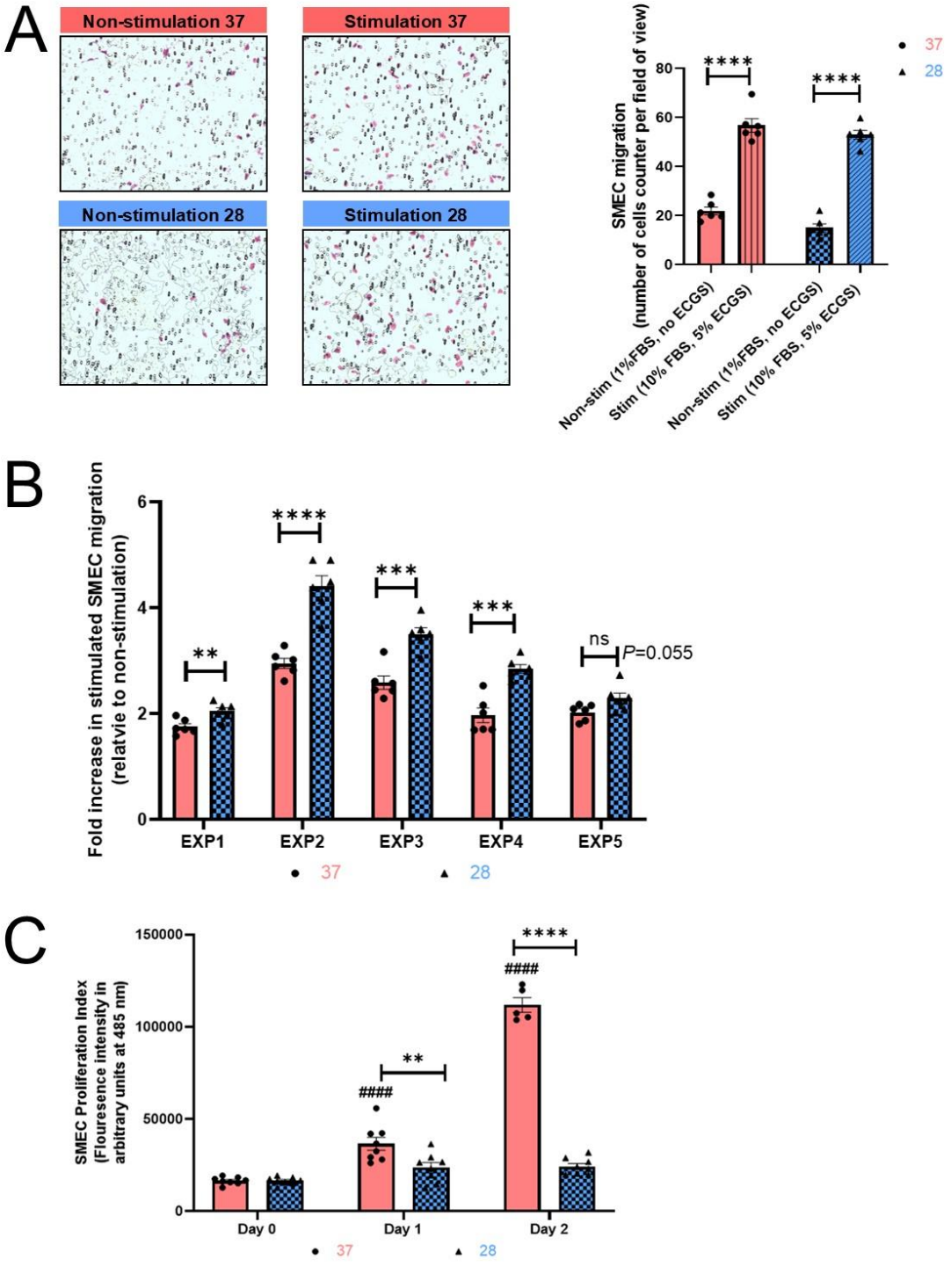

A
Non-stimulation 37
Stimulation 37
Non-stimulation 28
Stimulation 28
SMEC migration (number of cells counter per field of view)
37
28
****
****
Non-stim (1%FBS, no ECGS)
Stim (10% FBS, 5% ECGS)
Non-stim (1%FBS, no ECGS)
Stim (10% FBS, 5% ECGS)
B
Fold increase in stimulated SMEC migration (relatvie to non-stimulation)
**
****
***
***
ns
P=0.055
EXP1
EXP2
EXP3
EXP4
EXP5
37
28
C
SMEC Proliferation Index (Flouresence intensity in arbitrary units at 485 nm)
**
****
####
####
Day 0
Day 1
Day 2
37
28

Figure 15. Effect of cold stress preconditioning on skeletal muscle endothelial cell migratory activity.

(A) Representative images and histogram for skeletal muscle endothelial cells (SMECs) migration in Boyden chamber chemotaxis assay in response to non-stimulation (1% FBS, no ECGS) or stimulation (10% FBS, 5% ECGS) for 6h following 16h of thermal preconditioning (37 or 28). Migration is expressed as number of cells counter per field of view. Data presented is a single representative experiment of five independent experiments each with n=6 counts per condition (represented as means ± SEM). Significant difference between groups: ****, $P \leq 0.0001$. **(B)** Graphical representation comparing stimulated SMECs migratory activity from respective thermal preconditioning (37 or 28) in five independent experiments conducted. Data is expressed as a fold increase in stimulated migration relative to non-stimulatory conditions (Stim migration / Non-stim migration) (represented as means ± SEM with n=6 samples per condition). Each experiment is compared statistically by un-paired t-test. Significant difference between groups: **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. **(C)** Representative histogram of SMECs proliferation for up to two days (Day 0-2) in either control (37) or cold stress (28) conditions. Data presented is a single conducted experiment that is representative of three independent experiments each with n=4-8 samples per condition and exposure (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$; ****, $P \leq 0.0001$. Significant difference in comparison to Day 0: ####, $P \leq 0.0001$.

FIGURE 16

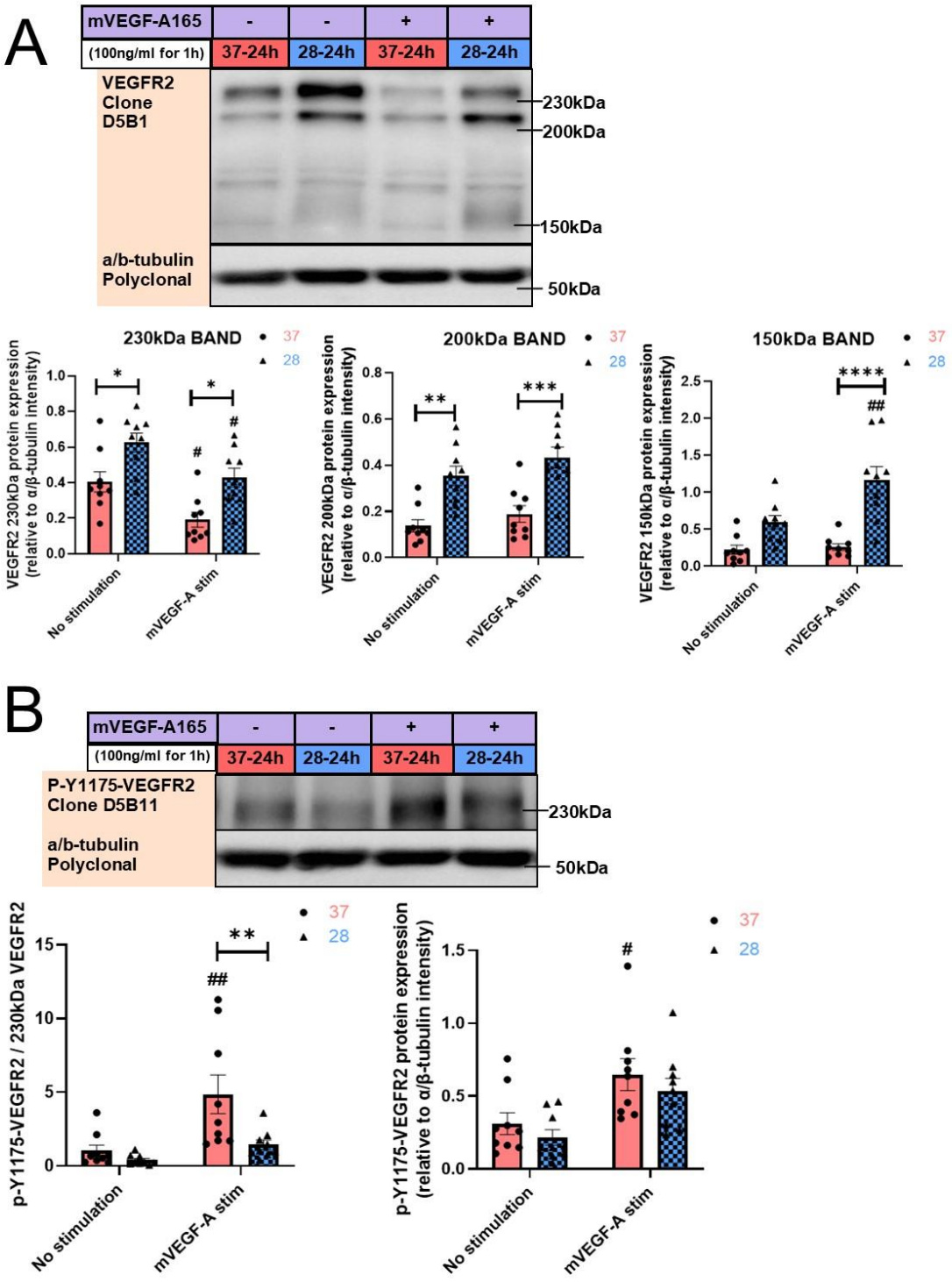

A
mVEGF-A165
(100ng/ml for 1h)
37-24h
28-24h
VEGFR2
Clone
D5B1
230kDa
200kDa
150kDa
a/b-tubulin
Polyclonal
50kDa
230kDa BAND
200kDa BAND
150kDa BAND
37
28
VEGFR2 230kDa protein expression
(relative to α/β-tubulin intensity)
VEGFR2 200kDa protein expression
(relative to α/β-tubulin intensity)
VEGFR2 150kDa protein expression
(relative to α/β-tubulin intensity)
No stimulation
mVEGF-A stim
B
mVEGF-A165
(100ng/ml for 1h)
37-24h
28-24h
P-Y1175-VEGFR2
Clone D5B11
a/b-tubulin
Polyclonal
230kDa
50kDa
p-Y1175-VEGFR2 / 230kDa VEGFR2
p-Y1175-VEGFR2 protein expression
(relative to α/β-tubulin intensity)
No stimulation
mVEGF-A stim

Figure 16. Effect of cold stress on vascular endothelial growth factor-receptor 2 expression and responsiveness to recombinant vascular endothelial growth factor-A stimulation.

(A) Representative immunoblots and densitometry analysis for protein expression of VEGF-receptor 2 (VEGFR2) in skeletal muscle endothelial cells preconditioned to control (37) or cold stress (28) conditions for 24h prior to stimulation with recombinant murine VEGF-A (mVEGF-A165) for 1h (100ng/ml). α/β-tubulin was used as a loading control. Data shown is a combined representation of three independent experiments conducted each with n=3 samples per condition of temperature and stimulation (represented as means ± SEM). Significant difference between groups: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Significant effect of mVEGF-A165 stimulation: #, $P \leq 0.05$; ##, $P \leq 0.01$. **(B)** Representative immunoblots and densitometry analysis for protein expression of phosphorylated VEGFR2 at tyrosine 1175 (p-Y1175-VEGFR2) in SMECs preconditioned to control (37) or cold stress (28) conditions for 23h prior to stimulation with recombinant murine VEGF-A (mVEGF-A165) for 1h (100ng/ml). α/β-tubulin was used as a loading control. Left panel shows p-Y1175-VEGFR2 protein expression when normalized to respective 230 kDa VEGFR2 band intensity. Right panel shows p-Y1175-VEGFR2 protein expression when normalized to α/β-tubulin intensity. Data shown is a combined representation of three independent experiments conducted each with n=3 samples per condition of temperature and stimulation (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$. Significant effect of mVEGF-A165 stimulation: #, $P \leq 0.05$; ##, $P \leq 0.01$.

FIGURE 17

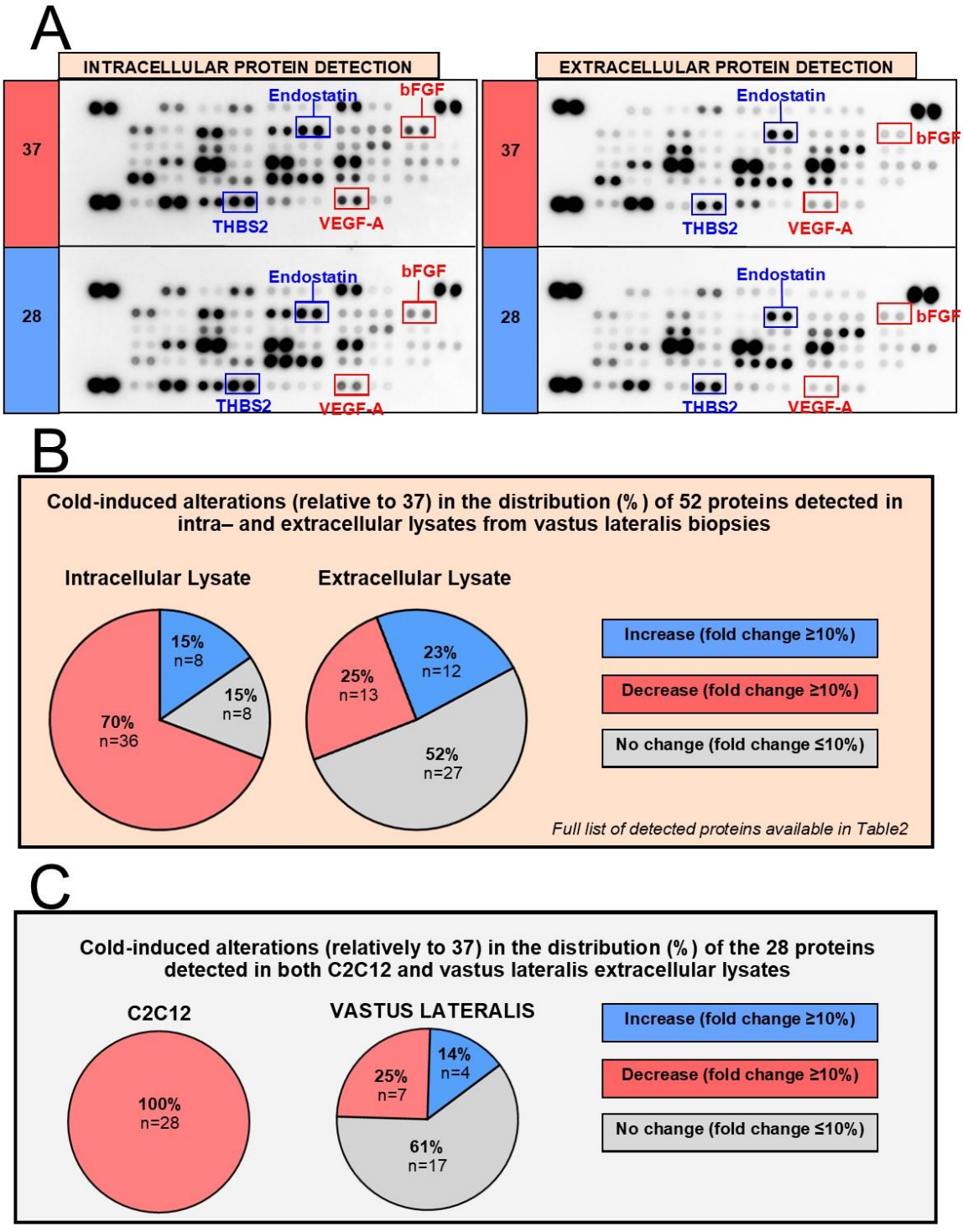

A
INTRACELLULAR PROTEIN DETECTION
EXTRACELLULAR PROTEIN DETECTION
37
28
Endostatin
bFGF
THBS2
VEGF-A
B
Cold-induced alterations (relative to 37) in the distribution (%) of 52 proteins detected in intra– and extracellular lysates from vastus lateralis biopsies
Intracellular Lysate
Extracellular Lysate
15% n=8
15% n=8
70% n=36
23% n=12
25% n=13
52% n=27
Increase (fold change ≥10%)
Decrease (fold change ≥10%)
No change (fold change ≤10%)
Full list of detected proteins available in Table2
C
Cold-induced alterations (relatively to 37) in the distribution (%) of the 28 proteins detected in both C2C12 and vastus lateralis extracellular lysates
C2C12
VASTUS LATERALIS
100% n=28
14% n=4
25% n=7
61% n=17
Increase (fold change ≥10%)
Decrease (fold change ≥10%)
No change (fold change ≤10%)

Figure 17. Effect of cold stress on 53 angiogenic molecules in intra- and extracellular lysates from mouse vastus lateralis muscle biopsies following muscle ex-vivo incubation.

A) Representative proteome profiler immunoblotting membranes for intra- and extracellular lysates from mouse vastus lateralis muscle biopsies incubated for 24h in control (37) or cold stress (28) conditions (EMI model). The anti-angiogenic endostatin and thrombospondin-2 (THBS2) and the pro-angiogenic vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF) are highlighted. Each highlighted square shows duplicate spots for a given protein. **(B)** Distribution in percentage of cold-induced alterations in proteins detected in the proteome profiler array in intracellular and extracellular EMI muscle lysates. **(C)** Distribution in percentage of cold-induced alterations in the 28 proteins detected in both C2C12 (Figure 13) and EMI muscle extracellular lysates.

Table 1

Proteins detected in C2C12 intracellular lysate	*Cold-induced changes (in % versus 37)*	*Proteins detected in C2C12 extracellular lysate*	*Cold-induced changes (in % versus 37)*
ADAMTS-1	-7.01	Angiopoietin-1	-30.01
Angiopoietin-1	+29.27	Angiopoietin-3	-25.44
Angiopoietin-3	-29.13	CXCL16	-25.25
Coagulation factor-3	-0.80	Endostatin	-45.94
Cyr61	+6.81	Endothelin-1	-18.10
Endostatin	+29.68	bFGF	-30.34
Endothelin-1	-7.46	CX3CL1	-27.35
bFGF	+0.08	HGF	-25.45
HB-EGF	-24.85	IGFBP-2	-15.79
IGFBP-2	+45.06	IGFBP-3	-41.93
NOV	-10.01	IL-1a	-32.82
Osteopontin	-0.73	MCP-1	-42.28
PDGF-A/A	-6.45	MMP-8	-28.55
PDGF-A/B	-16.73	MMP-9	-37.62
PlGF-2	-35.35	NOV	-48.02
CXCL12	+10.08	Osteopontin	-18.51
Serpin E1	+3.72	PDGF-A/A	-45.23
Serpin F1	-25.91	PDGF-A/B	-42.63
THBS2	+81.70	PlGF-2	-60.16
TIMP-1	+29.64	Prolactin	-37.68
VEGF-A	-5.67	Proliferin	-35.78
VEGF-B	-41.43	CXCL12	-20.50
		Serpin E1	-24.32
		Serpin F1	-40.11
		THBS2	-42.76
		TIMP-1	-17.17
		VEGF-A	-50.04
		VEGF-B	-38.71

Colour coding
Change ≤10%
Increase (fold change ≥10%)
Decrease (fold change ≥10%)

Table 2

Proteins detected in vastus lateralis intracellular lysate	*Cold-induced changes (in % versus 37)*	*Proteins detected in vastus lateralis extracellular lysate*	*Cold-induced changes (in % versus 37)*
ADAMTS-1	+35.53	ADAMTS-1	+34.64
Amphiregulin	+2.60	Amphiregulin	+6.79
Angiogenin	+33.42	Angiogenin	+13.25
Angiopoietin-1	+5.13	Angiopoietin-1	+1.11
Angiopoietin-3	-12.12	Angiopoietin-3	+3.28
Coagulation Factor 3	+20.35	Coagulation Factor 3	+6.01
CXCL16	-41.92	CXCL16	-5.19
Cyr61, IGFBP-10	-1.48	Cyr61, IGFBP-10	+36.35
DLL4	-28.01	DLL4	+7.58
CD26	-8.60	CD26	+3.62
EGF	-13.38	EGF	+6.95
Endoglin	+23.80	Endoglin	-10.40
Endostatin	+5.95	Endostatin	-8.27
Endothelin-1	-29.25	Endothelin-1	-1.58
aFGF	-41.55	aFGF	+14.85
bFGF	-35.49	bFGF	+21.22
KGF, FGF-7	-28.84	KGF, FGF-7	+12.91
Fractalkine, CX3CL1	-26.46	Fractalkine, CX3CL1	+2.24
GM-CSF	-10.73	GM-CSF	-28.97
HB-EGF	-30.63	HB-EGF	+7.29
HGF	-26.11	HGF	+4.21
IGFBP-1	-30.93	IGFBP-1	+4.86
IGFBP-2	-42.61	IGFBP-2	+15.82
IGFBP-3	-22.51	IGFBP-3	-1.69
IL-1a	-55.25	IL-1a	-6.06
IL-10	-32.75	IL-10	-2.39
IP-10, CXCL10	+8.88	IP-10, CXCL10	-12.43
KC, CXCL1	-3.23	KC, CXCL1	+12.92
Leptin	-23.09	Leptin	+21.51

Table 2

MCP-1	+4.73	MCP-1	+0.26
MIP-1a	-20.11	MIP-1a	-11.11
MMP-3	-29.01	MMP-3	-16.34
MMP-8	-57.45	MMP-8	-20.70
MMP-9	-51.83	MMP-9	-13.64
NOV	-23.56	NOV	+10.48
Osteopontin	-66.70	Osteopontin	-52.63
PD-ECGF	-36.93	PD-ECGF	-6.63
PDGF-AA	-33.95	PDGF-AA	+6.77
PDGF-AB / PDGF-BB	-15.16	PDGF-AB / PDGF-BB	+3.29
Pentraxin-3	+42.00	Pentraxin-3	-10.95
Platelet Factor-4	+19.19	Platelet Factor-4	+36.39
PlGF-2	-51.82	PlGF-2	-55.79
Prolactin	-53.89	Prolactin	+1.22
Proliferin	-55.20	Proliferin	-3.51
SDF-1, CXCL12	-24.26	SDF-1, CXCL12	+2.85
Serpin E1, PAI-1	-21.54	Serpin E1, PAI-1	-48.45
Serpin F1, PEDF	+17.85	Serpin F1, PEDF	-2.34
THBS2	+32.32	THBS2	+29.59
TIMP-1	-56.96	TIMP-1	-46.31
TIMP-4	-35.57	TIMP-4	+3.37
VEGF-A	-30.68	VEGF-A	-34.59
VEGF-B	-57.66	VEGF-B	+0.74

Colour coding

Change ≤10%
Increase (fold change ≥10%)
Decrease (fold change ≥10%)

3.9. Discussion.

Cold stress has been identified as a pro-angiogenic stimulus enhancing the formation of novel muscle capillaries (Bae et al., 2003; Banchero et al., 1985; Deveci and Egginton, 2003, 2002; D'Souza et al., 2018; Duchamp et al., 1992; Heroux and St. Pierre, 1956; Santocildes et al., 2021; Sillau et al., 1980). At the molecular level, studies on skeletal muscle tissue have essentially focused on VEGF-A with discrepancy in reported results (Al-horani et al., 2019; D'Souza et al., 2018; Ihsan et al., 2014; Joo et al., 2016; Kim et al., 2005; Magalhães et al., 2020; Opichka et al., 2019; Shute et al., 2020; Zak et al., 2017). However, these studies present many confounding variables (aerobic versus resistance exercise, training status, photoperiod, nutritional status, local versus circulating signals, implication of the nervous system) making difficult to parse the exact contribution of cold stress *per se*. *In-vitro* studies conducted on skeletal myoblasts or myotubes have also been limited to VEGF-A (Krapf et al., 2021; Sugasawa et al., 2016). Although muscle cells represent with no doubt a major source of angiogenic molecules within the skeletal muscle tissue, endothelial cells are the building elements of new vessels. It is therefore surprising that no studies have investigated how cold stress could affect skeletal muscle endothelial cells. Therefore, for the first time in the field of vascular biology, I have used here an *in-vitro* approach to determine the influence of cold stress *per se* on skeletal muscle angio-adaptation. I have focused on the direct effect of cold stress on muscle cells and endothelial cells, respectively, as well as on the indirect impact of cold on endothelial cells via the release of myotube-derived angiogenic molecules. Furthermore, my *in-vitro* approach has ensured the mitigation of possible confounding *in-vivo* variables elicited by cold stress on other tissues such as motoneuron innervation, arteriolar vasomotricity, or circulating factors. Finally, I used an *ex-vivo* muscle incubation assay to determine if the results I observed *in-vitro* were reproduced in whole muscle vastus lateralis biopsy samples.

As mentioned previously, majority of studies have focused on describing the effect of cold stress on VEGF-A in muscle tissue and cells despite both THBS1 and VEGF-A being respectively identified as key anti- and pro- angiogenic factors. Due to this discrepancy, I have focused on describing the effect of cold stress on THBS1 expression in myotubes. During my thesis, I have for the first time described cold stress to stimulate both THBS1 mRNA and intracellular protein in myotubes. When exposing differentiated C2C12 myotubes to 24h of cold stress I observed a strong

and significant increase in THBS1 mRNA and intracellular protein expression. To my knowledge, only two studies have documented an increase of THBS1 mRNA (Nascimento et al., 2020) and protein (Zieger et al., 2011) to cold stress, in human muscle biopsy samples and human coronary endothelial cells, respectively. These studies were however very exploratory in nature with the use of transcriptomics and proteomics. Furthermore, by re-warming and re-cooling myotubes, I have shown that cold stress stimulated THBS1 protein expression is a physiological, reversible, and re-inducible response. This is similar to the reversible and re-inducible effect of hypoxia on *THBS1* gene expression described by Phelan and colleagues (Phelan et al., 1998).

Several studies have shown cold stress to stimulate VEGF-A gene and protein expression (Al-horani et al., 2019; Ihsan et al., 2014; Joo et al., 2016; Kim et al., 2005). However, because these studies were performed *in-vivo,* most often investigating the use of cold-water immersion as an ergogenic aid to exercise, it cannot be discerned whether these results are due to cold stress *per se* or confounding physiological responses known to stimulate VEGF-A production. Such confounding factors are an acute bout of exercise, increased shear stress, or elevated metabolic activity (Birot et al., 2003; Leick et al., 2009; Milkiewicz et al., 2001; Uchida et al., 2015; Zwetsloot et al., 2008). In comparison, the effect of cold stress *in-vitro* on VEGF-A protein in myotubes yet remains to be characterized. In my study, I observed cold stress to have no effect on intracellular VEGF-A protein expression in differentiated C2C12 myotubes both quantitatively and qualitatively, by ELISA and proteomics, respectively.

In previous work from our lab, we have used the VEGF-A / THBS1 ratio as a rudimentary indicator of the directionality of an angiogenic response (Aiken et al., 2019, 2016; Roudier et al., 2012, 2010). Whilst my intracellular results suggested cold stress to stimulate a preferentially anti-angiogenic molecular expression in differentiated C2C12 myotubes, my extracellular results did not reflect intracellular observations. When analyzing the extracellular lysates from differentiated C2C12 myotubes conditioned to cold stress for 24h, I observed a significant reduction in both THBS1 and VEGF-A protein. While cold stress was previously reported to reduce human myotube secretion of VEGF-A protein, *in-vitro* (Krapf et al., 2021), I was perplexed to observe a reduction in secreted THBS1 protein given the robust cold stress stimulated intracellular response. However, my proteomics results demonstrated that this differential intra- and extracellular expression was not exclusively THBS1-related. I have identified six additional proteins (Angiopoietin-1,

Endostatin, insulin like growth factor binding protein 2 (IGFBP2), C-X-C motif chemokine ligand 12 (CXCL12), THBS2, and tissue-inhibitor of metalloproteinases-1(TIMP-1)), that upon exposure to cold stress were upregulated at the intracellular level but decreased extracellularly. Similar results have been reported when exposing human primary myotubes to cold stress (18h at 18℃). Krapf and colleagues (Krapf et al., 2021) reported cold stress to stimulate a significant increase in intracellular interlukin-8 (IL-8) expression that was significantly reduced at the extracellular level. This would lead one to presume that the observed intracellular expression of these proteins may be due to retention. However, my data showing cold stress to increase THBS1 mRNA expression may refute this. Furthermore, I observed cold stress to reduce the expression of all twenty-eight proteins I detected in the extracellular lysate of differentiated C2C12 myotubes via proteomic screening. Of these twenty-eight proteins, six and five respectively, had no change or a reduction in expression at the intracellular level. Thus, the selective retention of specific proteins in response to cold stress seems unlikely, yet also intriguing for further research directions.

As mentioned above, endothelial cells are the primary constituents of novel capillaries, thus it is surprising that to my knowledge the effect of cold stress on SMECs angiogenic responses has yet to be described. When I preconditioned SMECs to 16h of cold stress, the magnitude of their migratory response in the chemotaxis assay was significantly greater in comparison to SMECs preconditioned at 37˚C. I also observed a complete attenuation of the proliferative capacity of cold conditioned SMECs in addition to an elevated expression of VEGFR2. The cold shock responsive protein RBM3 has been linked to mediating F-actin organization necessary for the adoption of cell polarity and has been shown to enhance cell migration (Mardakheh et al., 2015; Pilotte et al., 2018, 2009). Therefore, when I observed RBM3 and VEGFR2 to be elevated in cold stressed SMECs along with a reduced proliferation, I inferred that the possible mechanism for improved SMECs migration with cold stress preconditioning was due to an induction of a tip cell phenotype in SMECs. While this may suggest that cold stress is a pro-angiogenic stimulus by enhancing a migratory endothelial cell phenotype, I caution against this conclusion. For example, when I conditioned SMECs to cold stress I observed a reduction in phosphorylation of VEGFR2 at residue Y1175 when stimulating with recombinant VEGF-A. VEGFR2 phosphorylation at Y1175 is a key step for downstream MAPK/ERK cascades involved in endothelial cell proliferation (Takahashi et al., 2001; Wang et al., 2020). While endothelial cell migration is important, it is the proliferative action of stalk cells that drives the elongation of a vessel sprout.

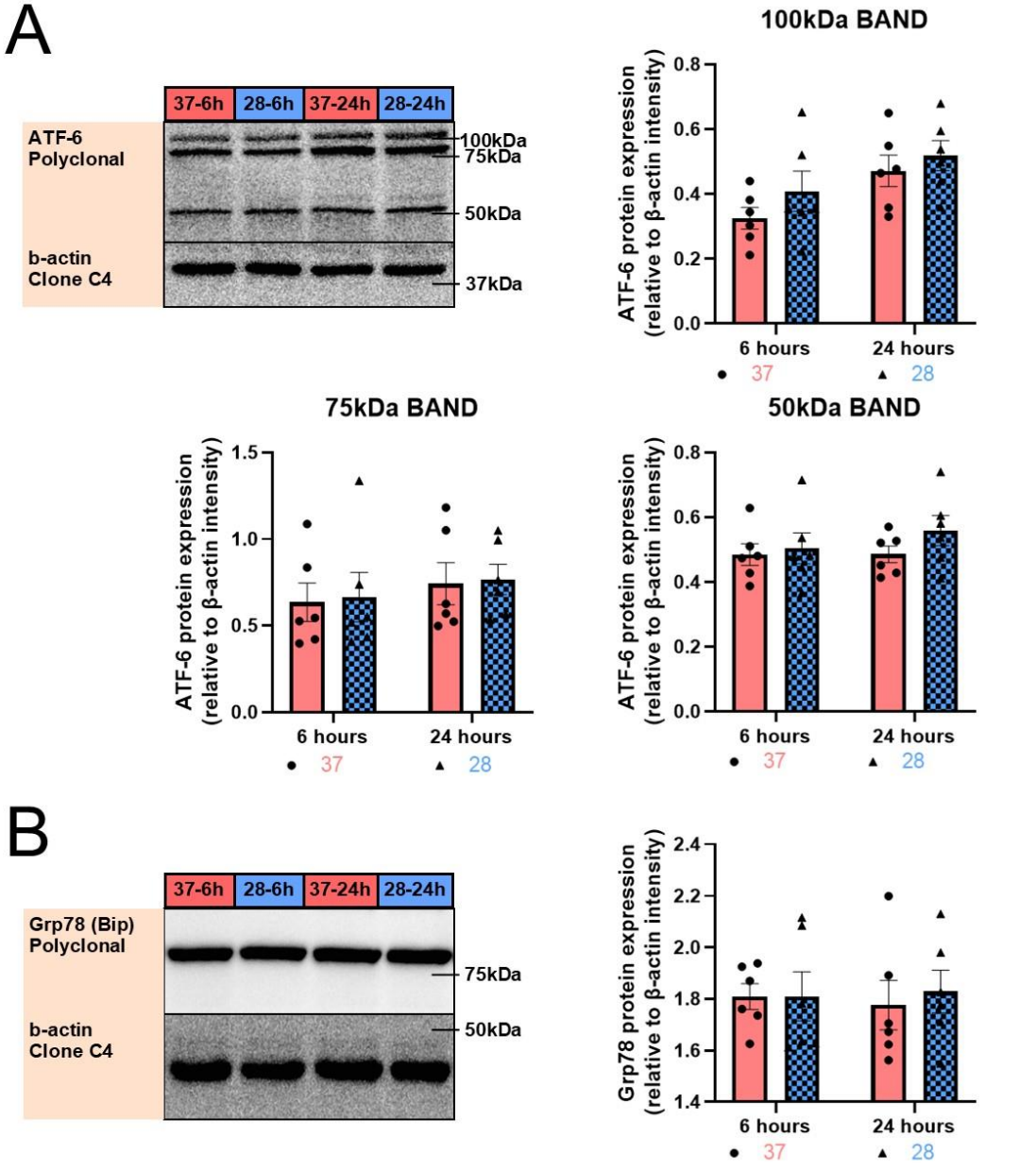
Therefore, while cold stress enhances the migratory aspect of sprouting angiogenesis, without a concomitant improvement in cell proliferation, it cannot be stated that cold stress would enhance sprouting angiogenesis relative to control conditions based on my observations *in-vitro*. Furthermore, it is important to note that in my study when assessing SMECs p-VEGFR2-Y1175 expression and proliferation, SMECs were maintained in constant cold stress. In comparison, when assessing SMECs migratory activity via the Boyden chamber chemotaxis assay. SMECs were preconditioned to cold stress and then challenged for their migratory capacity in normothermic conditions. This would then suggest that in fact it is cooling accompanied by a transient rewarming in which SMECs migration is enhanced. This begs the possibility of then using pre-cooling prior to acute exercise to enhance the angiogenic response of exercise. For example, an enhanced rate of VEGF-A secretion has been described in human myotubes preconditioned to cold stress with accompanied rewarming (Krapf et al., 2021). I have shown cold stress to elevated SMECs expression of VEGFR2 in conjunction with precooling to enhance the migratory responsiveness of SMECs. Therefore, pre-cooling prior to acute exercise may lead to an increased rate of VEGF-A secretion of the skeletal muscle post exercise; to a greater opportunity for this VEGF-A to interact with SMECs given their elevated VEGFR2 expression; and then to an enhanced ability of these SMECs to migrate towards angiogenic cues. In this sense, it would be intriguing to evaluate the possible use of localized muscle cooling prior to acute exercise to be used as an ergogenic aid to improve the angiogenic response of exercise training.

Finally, in my study I observed some discrepancy between the cold-induced expression of secreted proteins from C2C12 myotubes and those secreted from whole muscle vastus lateralis biopsies. Unlike with C2C12 myotubes, whole muscle biopsies exposed to cold do not have a complete attenuation of their secretory activity, with respect to angiogenic proteins. I have identified four proteins that were increased both in intracellular and extracellular lysates from whole muscle biopsies exposed to cold. Of these four proteins (a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS-1), Angiogenin, Platelet factor -4, Thrombospondin-2), three have anti-angiogenic activity. ADAMTS-1 is a secreted metalloproteinase that enhances THBS1 cleavage and subsequent anti-angiogenic activity in addition to directly binding to VEGF-A and dampening VEGFR2 signalling (Hohberg et al., 2011; Iruela-Arispe et al., 2003). Platelet factor -4 directly interferes with both VEGF-A, bFGF, and their respective receptors, reducing endothelial cell proliferation, adhesion, and migration (Bikfalvi, 2004; Maurer et al., 2006; Wang

and Huang, 2013). Finally, thrombospondin-2 (THBS2), has been shown to be a potent anti-angiogenic factor with similar functionality to THBS1 (Lawler and Lawler, 2012; Volpert et al., 1995). Furthermore, I have also shown that like with myotubes, THBS1 intracellular protein expression is stimulated by cold stress in whole muscle biopsy samples. Taken together these results may suggest that cold stress influences an anti- angiogenic microenvironment in whole muscle tissue.

In conclusion, I have used for the first time an original *in-vitro* approach to elucidate the influence of cold stress on skeletal muscle angio-adaptation. I have shown that cold stress stimulates the expression of the potent anti-angiogenic THBS1 in both differentiated C2C12 myotubes and whole muscle vastus lateralis biopsies. Yet, I was unable to come to a consensus regarding the angiogenic microenvironment produced by cold conditioned differentiated C2C12 myotubes due to an attenuation of their secretion of both pro- and anti- angiogenic factors. In comparison, I have identified that direct cold stress exposure improved skeletal muscle endothelial cells migratory response, thus suggesting a possible pro- angiogenic effect of cold on these cells. However, I have described cold stress to stimulate the production and release of potent anti-angiogenic factors in whole muscle tissue biopsies, raising the question of the participation of additional cell types such as pericytes for example. Altogether, my results demonstrate the complexity of skeletal muscle angio-adaptation in response to cold stress and opens the door to future research.

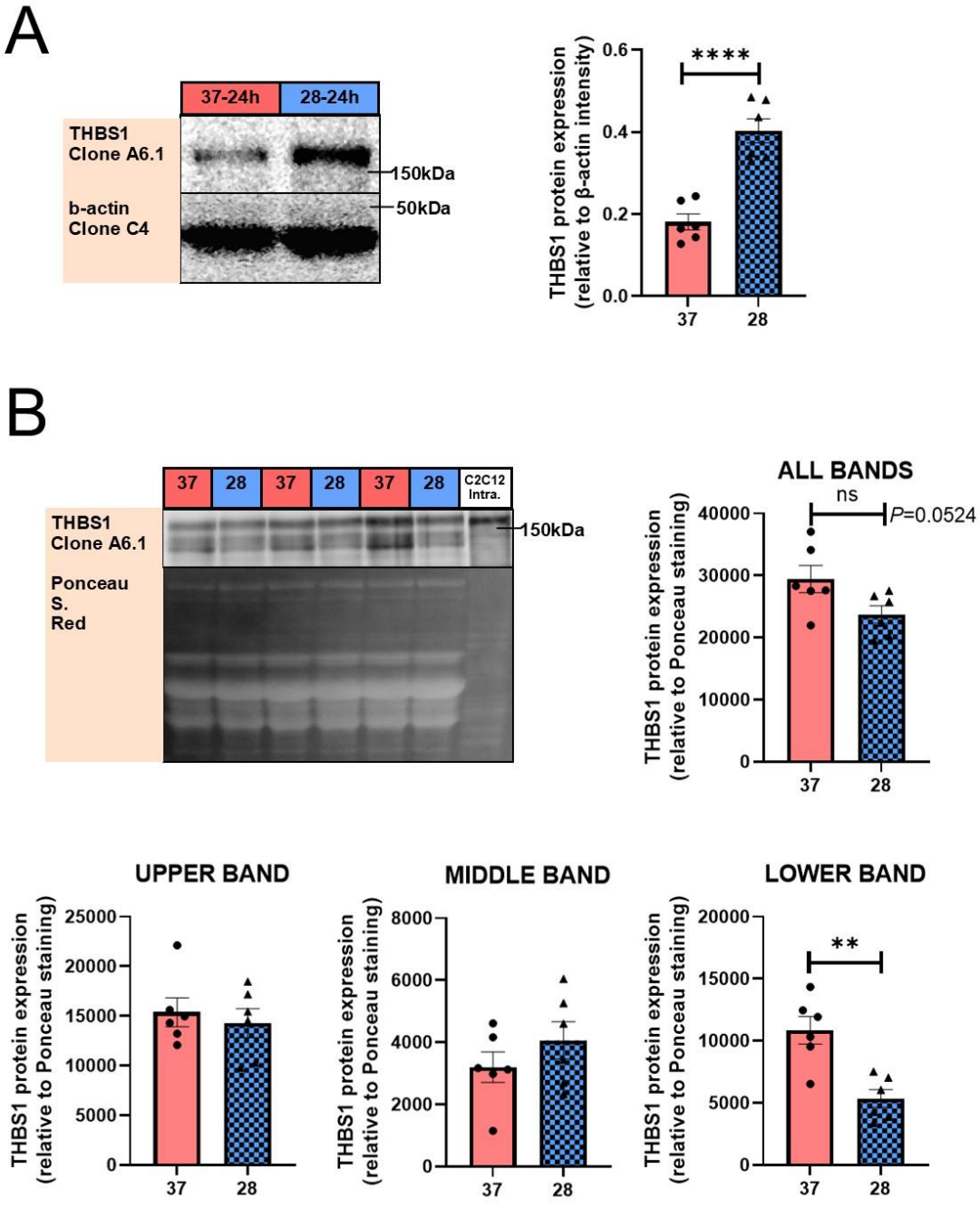
Supplemental FIGURE 1



Supplemental Figure 1. Effect of cold stress on activating transcription factor 6 and glucose regulated protein 78 in differentiated C2C12 myotubes.

(A) Representative immunoblots and densitometry analysis for intracellular protein expression of activating transcription factor 6 (ATF6) in differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 6h and 24h. β-actin was used as a loading control. Data shown is a single representative experiment of three independently conducted experiments with n=6 samples per condition of exposure time and temperature (represented as means ± SEM). **(B)** Representative immunoblots and densitometry analysis for intracellular protein expression of glucose regulated protein 78 (Grp78) in differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 6h and 24h. β-actin was used as a loading control. Data shown is a single representative experiment of three independently conducted experiments with n=6 samples per condition of exposure time and temperature (represented as means ± SEM).

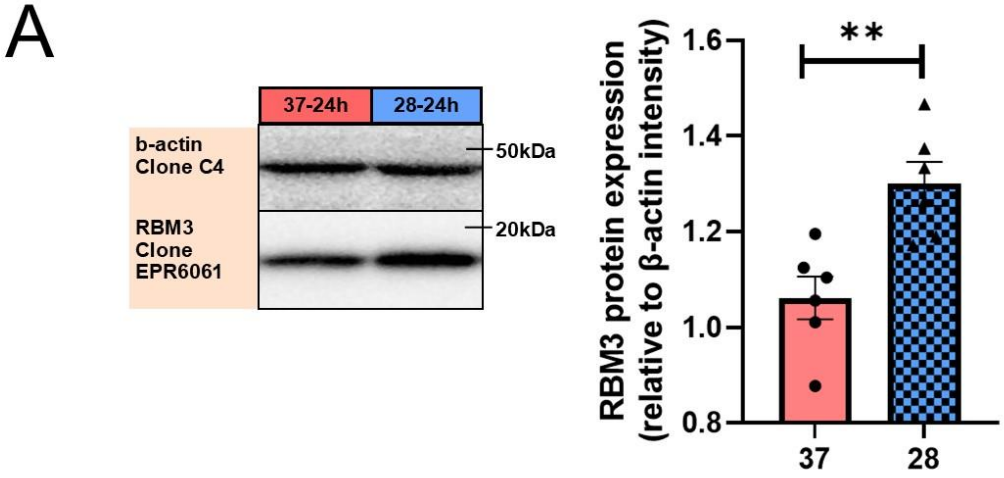
Supplemental FIGURE 2



Supplemental Figure 2. Effect of cold stress on intra- and extracellular thrombospondin-1 expression in differentiated C2C12 myotubes used to characterize vascular endothelial growth factor-A protein expression.

(A) Representative immunoblots and densitometry analyses for thrombospondin-1 (THBS1) protein expression in differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. β-actin was used as a loading control. Data shown is from a single conducted experiment with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: ****, $P \leq 0.0001$. **(B)** Representative immunoblots and densitometry analyses for THBS1 protein expression in extracellular lysate from differentiated C2C12 myotubes exposed to control (37) or cold stress (28) conditions for 24h. Ponceau S Red staining was used to ensure equal protein loading. Data shown is from a single conducted experiment with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$.

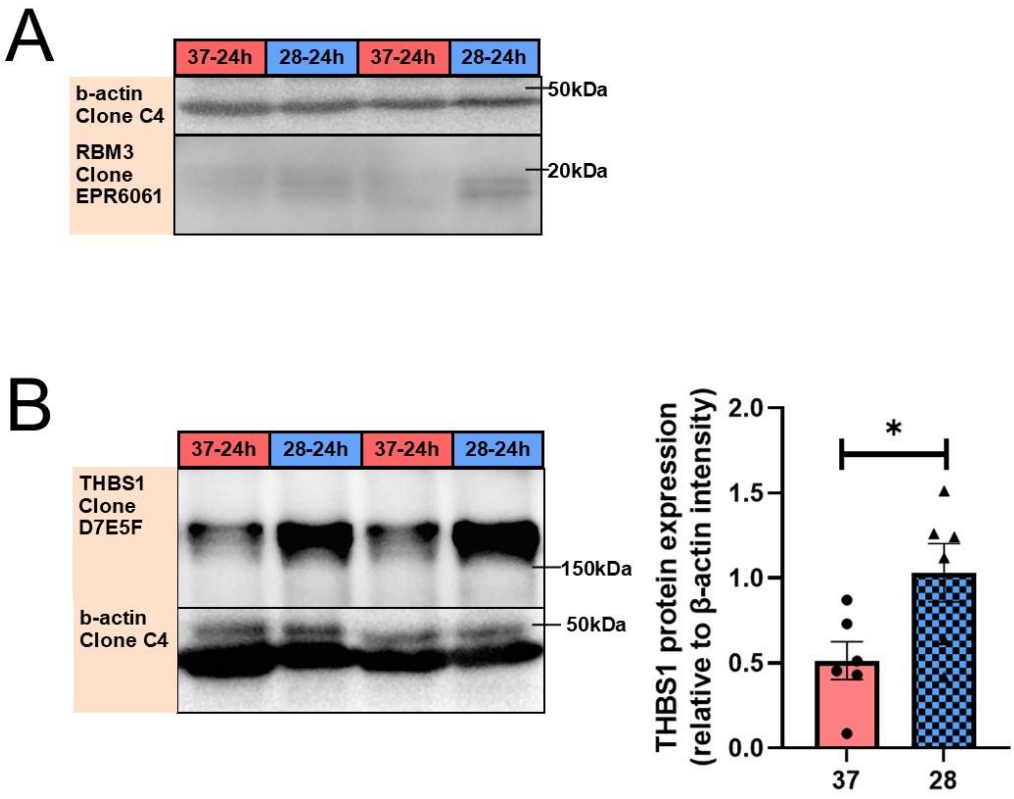
Supplemental FIGURE 3



Supplemental Figure 3. Effect of cold stress on RNA-binding motif 3 protein expression in skeletal muscle endothelial cells.

(A) Representative immunoblots and densitometry analyses for intracellular cold-shock RNA-binding motif 3 (RBM3) protein expression in SMECs exposed to 24h of control (37) or cold stress conditions (28). β-actin was used as a loading control. Data shown is a single representative experiment of two independently conducted experiments with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: **, $P \leq 0.01$.

Supplemental FIGURE 4



Supplemental Figure 4. Effect of cold stress on RNA-binding motif 3 and thrombospondin-1 protein expression in ex-vivo muscle incubation mouse vastus lateralis muscle biopsy.

(A) Representative immunoblots for the cold-shock RNA-binding motif 3 protein (RBM3) in mouse vastus lateralis muscle biopsies incubated for 24h *in-vitro* in control (37) or cold stress (28) conditions (EMI assay). β-actin was used as a loading control. **(B)** Representative immunoblots and densitometry analysis of thrombospondin-1 (THBS1) protein expression in mouse vastus lateralis muscle biopsies incubated for 24h *in-vitro* in control (37) or cold stress (28) conditions (EMI assay). β-actin was used as a loading control. Data shown is from a single conducted experiment with n=6 samples per condition (represented as means ± SEM). Significant difference between groups: *, $P \leq 0.05$.

4. CONCLUSION

Perturbations in skeletal muscle blood flow demand elicit functional adaptations in the muscle capillary microvasculature. These adaptations can either include the growth (angiogenesis) or the rarefaction of capillaries. Skeletal muscle angio-adaptation refers to dynamic and coordinated processes at molecular and cellular levels that influence muscle capillary remodelling.

The use of the environmental stressors such as ambient hypoxia or cold stress (mainly via cold-water immersion protocols) has gained popularity in both sport performance and therapeutic settings in order to potentiate exercise-induced adaptations to skeletal muscle (Broatch et al., 2018; Girard et al., 2020; Girard and Chalabi, 2013; Girard and Pluim, 2013; Ihsan et al., 2021; Millet et al., 2016, 2010; Verges et al., 2015). In the case of hypoxia, the main goal is the superimposition of an ambient hypoxic stress and exercise-induced tissular hypoxia to exacerbate the level of hypoxic stress experienced by the skeletal muscle (Favier et al., 2015). In the context of cold-water immersion, cold stress stimulation is often used post-exercise to attenuate training-induced decrements in muscle strength, aerobic performance, muscle damage, inflammation, muscle soreness, and perceptions of fatigue, in hopes of improving subsequent training performance (Broatch et al., 2018; Ihsan et al., 2021). Furthermore, high altitude represents a unique situation in which there is a combination of the stressors.

Interestingly, both environmental stressors display the capability to affect skeletal muscle angio-adaptive processes and in turn the plasticity of the muscle capillaries both with and without a concomitant exercise stressor. Through this thesis, I have identified many similarities with respect to gaps of knowledge and controversies between ambient hypoxia and cold stress regarding skeletal muscle angio-adaptation. First, both environmental stressors lead to an improved muscle capillary density, however it is debated whether this occurs due to the formation of novel capillaries. Second, in both fields many studies have measured VEGF-A mRNA from vastus lateralis biopsies and have concluded changes in VEGF-A expression to be synonymous with a comprehensive angio-adaptive response. Third, despite THBS1 being identified as a key anti-angiogenic molecule, there is much less original research describing the effect of either environmental stressor on its expression, relative to VEGF-A.

Of the two components of this thesis, **study 1** was a comprehensive review of the literature regarding the effect of ambient, and exercise-induced hypoxia, both respectively and combined,

on skeletal muscle angio-adaptation. After completing the review, a lack of consensus was found regarding the impact of ambient hypoxia to stimulate the formation of novel muscle capillaries. However, ambient hypoxia *per se* does influence skeletal muscle capillarization by inducing myofiber atrophy. In comparison, ambient hypoxia combined with exercise potentiates the angiogenic effect of exercise on muscle angiogenesis leading to greater improvements in skeletal muscle capillarization than exercise alone. Finally, majority of studies assessing the impact of ambient hypoxia with or without exercise on molecular aspects of skeletal muscle angio-adaptation focused on the characterization of VEGF-A. Yet, due to differences in methodological approach it was difficult to synthesize these studies into a concise take-home message. Furthermore, the focus of existing literature on VEGF-A highlighted a true discrepancy in original research concerning the sensitivity of other molecules relevant to skeletal muscle angio-adaptation and demonstrated current gaps of knowledge in the field.

In **study 2**, a novel *in-vitro* approach was taken to assess the influence of cold stress *per se* on skeletal muscle angio-adaptation. Specifically, the impact of cold stress on differentiated C2C12 myotubes and their paracrine activity, as well as the effect of cold on murine skeletal muscle endothelial cells (SMECs) and whole muscle biopsy samples were investigated. In this study it was identified that cold stress stimulates the production of the potent anti-angiogenic THBS1 in differentiated C2C12 myotubes. Yet, proteomic and Boyden chamber migration results together did not enable me to reach any consensus on whether cold stress stimulated a pro-angiogenic microenvironment. In comparison, cold stress appeared as a potential pro-angiogenic stressor in SMECs by influencing their migratory response. Furthermore, cold stress stimulated an increased expression of VEGF-receptor 2 (VEGFR2) in these cells. In conjunction, these two observations in cold-conditioned SMECs led to the postulation of muscle pre-cooling prior to exercise to potentiate the angiogenic effect of exercise. Finally, whole muscle biopsy samples exposed to cold stress via an ex-vivo muscle incubation assay showed cold stress to potentially promote an anti-angiogenic microenvironment as evidenced by proteomic results.

In conclusion, my thesis has sought to evaluate the impact of environmental stressors on skeletal muscle angio-adaptation, specifically ambient hypoxia, and cold stress. With the comprehensive review, valuable skills to a young researcher were learned by synthesizing existing literature into written, and graphical, un-biased evaluations. In comparison, the original research

component of this thesis allowed for the generation of novel observations based on a scientific question. However, these novel observations have cultivated more scientific questions left unanswered. For example, what is the influence of extracellular lysate from electro-stimulated differentiated C2C12 myotubes that undergo recovery in cold stress conditions on the migratory activity of cold pre-conditioned SMECs? Or what is the effect of cold stress *in-vivo*, on the angiogenic microenvironment produced by skeletal muscle and are these conditions altered in diseased states such as obesity and type 1 or type 2 diabetes? Regardless, it is clear there are many gaps of knowledge and existing opportunities for future research directions in the field of environmental muscle physiology. I am excited by the opportunity to continue to pursue these future research directions at the PhD level in Dr.Birot's lab.

References

Abdelmoez, A.M., Sardón Puig, L., Smith, J.A.B., Gabriel, B.M., Savikj, M., Dollet, L., Chibalin, A.V., Krook, A., Zierath, J.R., Pillon, N.J., 2020. Comparative profiling of skeletal muscle models reveals heterogeneity of transcriptome and metabolism. Am. J. Physiol.-Cell Physiol. 318, C615–C626. https://doi.org/10.1152/ajpcell.00540.2019

Adair, T.H., Montani, J.-P., 2010. Angiogenesis, Integrated Systems Physiology: from Molecule to Function to Disease. Morgan & Claypool Life Sciences, San Rafael (CA).

Aiken, J., Mandel, E.R., Riddell, M.C., Birot, O., 2019. Hyperglycaemia correlates with skeletal muscle capillary regression and is associated with alterations in the murine double minute-2/forkhead box O1/thrombospondin-1 pathway in type 1 diabetic BioBreeding rats. Diab. Vasc. Dis. Res. 16, 28–37. https://doi.org/10.1177/1479164118805928

Aiken, J., Roudier, E., Ciccone, J., Drouin, G., Stromberg, A., Vojnovic, J., Olfert, I.M., Haas, T., Gustafsson, T., Grenier, G., Birot, O., 2016. Phosphorylation of murine double minute-2 on Ser ^{166} is downstream of VEGF-A in exercised skeletal muscle and regulates primary endothelial cell migration and *FoxO* gene expression. FASEB J. 30, 1120–1134. https://doi.org/10.1096/fj.15-276964

Al-horani, R.A., Al-Trad, B., Haifawi, S., 2019. Modulation of cardiac vascular endothelial growth factor and PGC-1α with regular postexercise cold-water immersion of rats. J. Appl. Physiol. 126, 1110–1116. https://doi.org/10.1152/japplphysiol.00918.2018

Amadio, M., Scapagnini, G., Lupo, G., Drago, F., Govoni, S., Pascale, A., 2008. PKCbetaII/HuR/VEGF: A new molecular cascade in retinal pericytes for the regulation of VEGF gene expression. Pharmacol. Res. 57, 60–66. https://doi.org/10.1016/j.phrs.2007.11.006

Ameln, H., Gustafsson, T., Sundberg, C.J., Okamoto, K., Jansson, E., Poellinger, L., Makino, Y., 2005. Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. FASEB J. 19, 1009–1011. https://doi.org/10.1096/fj.04-2304fje

Amouzou, C., Breuker, C., Fabre, O., Bourret, A., Lambert, K., Birot, O., Fédou, C., Dupuy, A.-M., Cristol, J.-P., Sutra, T., Molinari, N., Maimoun, L., Mariano-Goulart, D., Galtier, F., Avignon, A., Stanke-Labesque, F., Mercier, J., Sultan, A., Bisbal, C., 2016. Skeletal Muscle Insulin Resistance and Absence of Inflammation Characterize Insulin-Resistant Grade I Obese Women. PloS One 11, e0154119. https://doi.org/10.1371/journal.pone.0154119

Andersen, P., 1975. Capillary Density in Skeletal Muscle of Man. Acta Physiol. Scand. 95, 203–205. https://doi.org/10.1111/j.1748-1716.1975.tb10043.x

Andersen, P., Henriksson, J., 1977. Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. J. Physiol. 270, 677–690. https://doi.org/10.1113/jphysiol.1977.sp011975

Angleys, H., Østergaard, L., 2020. Krogh's capillary recruitment hypothesis, 100 years on: Is the opening of previously closed capillaries necessary to ensure muscle oxygenation during

exercise? Am. J. Physiol.-Heart Circ. Physiol. 318, H425–H447. https://doi.org/10.1152/ajpheart.00384.2019

Armstrong, R.B., Ianuzzo, C.D., Laughlin, M.H., 1986. Blood flow and glycogen use in hypertrophied rat muscles during exercise. J. Appl. Physiol. Bethesda Md 1985 61, 683–687. https://doi.org/10.1152/jappl.1986.61.2.683

Asano, Kaneoka, Nomura, Asano, Sone, Tsurumaru, Yamashita, Matsuo, Suzuki, Okuda, 1998. Increase in serum vascular endothelial growth factor levels during altitude training. Acta Physiol. Scand. 162, 455–459. https://doi.org/10.1046/j.1365-201X.1998.0318e.x

Audet, G.N., Fulks, D., Stricker, J.C., Olfert, I.M., 2013. Chronic Delivery of a Thrombospondin-1 Mimetic Decreases Skeletal Muscle Capillarity in Mice. PLoS ONE 8, e55953. https://doi.org/10.1371/journal.pone.0055953

Auerbach, R., Alby, L., Morrissey, L.W., Tu, M., Joseph, J., 1985. Expression of organ-specific antigens on capillary endothelial cells. Microvasc. Res. 29, 401–411. https://doi.org/10.1016/0026-2862(85)90028-7

Bae, K.A., An, N.Y., Kwon, Y.W., Kim, C., Yoon, C.S., Park, S.C., Kim, C.K., 2003. Muscle fibre size and capillarity in Korean diving women: **Cold-water exposure and muscle morphology**. Acta Physiol. Scand. 179, 167–172. https://doi.org/10.1046/j.1365-201X.2003.01185.x

Bahtiyar, M.O., Buhimschi, C., Ravishankar, V., Copel, J., Norwitz, E., Julien, S., Guller, S., Buhimschi, I.A., 2007. Contrasting effects of chronic hypoxia and nitric oxide synthase inhibition on circulating angiogenic factors in a rat model of growth restriction. Am. J. Obstet. Gynecol. 196, 72.e1-72.e6. https://doi.org/10.1016/j.ajog.2006.07.048

Ballak, S.B., Busé-Pot, T., Harding, P.J., Yap, M.H., Deldicque, L., de Haan, A., Jaspers, R.T., Degens, H., 2016. Blunted angiogenesis and hypertrophy are associated with increased fatigue resistance and unchanged aerobic capacity in old overloaded mouse muscle. Age Dordr. Neth. 38, 39. https://doi.org/10.1007/s11357-016-9894-1

Banchero, N., 1985. Adaptation of muscle capillarity. Adv. Exp. Med. Biol. 191, 355–363. https://doi.org/10.1007/978-1-4684-3291-6_36

Banchero, N., Gimenez, M., Rostami, A., Eby, S.H., 1976. Effects of simulated altitude on O2 transport in dogs. Respir. Physiol. 27, 305–321. https://doi.org/10.1016/0034-5687(76)90060-8

Banchero, N., Kayar, S.R., Lechner, A.J., 1985. Increased capillarity in skeletal muscle of growing guinea pigs acclimated to cold and hypoxia. Respir. Physiol. 62, 245–255. https://doi.org/10.1016/0034-5687(85)90118-5

Barcroft, H., Edholm, O.G., 1946. Temperature and blood flow in the human forearm. J. Physiol. 104, 366–376. https://doi.org/10.1113/jphysiol.1946.sp004129

Bauer, P.M., Bauer, E.M., Rogers, N.M., Yao, M., Feijoo-Cuaresma, M., Pilewski, J.M., Champion, H.C., Zuckerbraun, B.S., Calzada, M.J., Isenberg, J.S., 2012. Activated CD47

promotes pulmonary arterial hypertension through targeting caveolin-1. Cardiovasc. Res. 93, 682–693. https://doi.org/10.1093/cvr/cvr356

Baum, O., Jentsch, L., Odriozola, A., Tschanz, S.A., Olfert, I.M., 2017. Ultrastructure of Skeletal Muscles in Mice Lacking Muscle-Specific VEGF Expression. Anat. Rec. Hoboken NJ 2007 300, 2239–2249. https://doi.org/10.1002/ar.23644

Belloni, P.N., Nicolson, G.L., 1988. Differential expression of cell surface glycoproteins on various organ-derived microvascular endothelia and endothelial cell cultures. J. Cell. Physiol. 136, 398–410. https://doi.org/10.1002/jcp.1041360303

Bhutta, B.S., Alghoula, F., Berim, I., 2022. Hypoxia, in: StatPearls. StatPearls Publishing, Treasure Island (FL).

Bigard, A.X., Brunet, A., Guezennec, C.Y., Monod, H., 1991. Effects of chronic hypoxia and endurance training on muscle capillarity in rats. Pflugers Arch. 419, 225–229. https://doi.org/10.1007/BF00371099

Bikfalvi, A., 2004. Platelet factor 4: an inhibitor of angiogenesis. Semin. Thromb. Hemost. 30, 379–385. https://doi.org/10.1055/s-2004-831051

Birot, O.J.G., Koulmann, N., Peinnequin, A., Bigard, X.A., 2003. Exercise-induced expression of vascular endothelial growth factor mRNA in rat skeletal muscle is dependent on fibre type. J. Physiol. 552, 213–221. https://doi.org/10.1113/jphysiol.2003.043026

Birot, O.J.G., Peinnequin, A., Simler, N., Cuyck-Gandré, H. van, Hamel, R., Bigard, X.A., 2004. Vascular endothelial growth factor expression in heart of rats exposed to hypobaric hypoxia: Differential response between mRNA and protein: VEGF IN HEART DURING CHRONIC AMBIENT HYPOXIA. J. Cell. Physiol. 200, 107–115. https://doi.org/10.1002/jcp.20002

Boegehold, M.A., Johnson, P.C., 1988. Periarteriolar and tissue PO2 during sympathetic escape in skeletal muscle. Am. J. Physiol. 254, H929-936. https://doi.org/10.1152/ajpheart.1988.254.5.H929

Bonaterra, G.A., Then, H., Oezel, L., Schwarzbach, H., Ocker, M., Thieme, K., Di Fazio, P., Kinscherf, R., 2016. Morphological Alterations in Gastrocnemius and Soleus Muscles in Male and Female Mice in a Fibromyalgia Model. PLOS ONE 11, e0151116. https://doi.org/10.1371/journal.pone.0151116

Boos, C.J., Lamb, C.M., Midwinter, M., Mellor, A., Woods, D.R., Howley, M., Stansfield, T., Foster, M., O'Hara, J.P., 2018. The Effects of Acute Hypoxia on Tissue Oxygenation and Circulating Alarmins in Healthy Adults. Physiol. Res. 935–943. https://doi.org/10.33549/physiolres.933743

Booth, F.W., Thomason, D.B., 1991. Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. Physiol. Rev. 71, 541–585. https://doi.org/10.1152/physrev.1991.71.2.541

Breen, E., Tang, K., Olfert, M., Knapp, A., Wagner, P., 2008. Skeletal muscle capillarity during hypoxia: VEGF and its activation. High Alt. Med. Biol. 9, 158–166. https://doi.org/10.1089/ham.2008.1010

Breen, E.C., Johnson, E.C., Wagner, H., Tseng, H.M., Sung, L.A., Wagner, P.D., 1996. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J. Appl. Physiol. 81, 355–361. https://doi.org/10.1152/jappl.1996.81.1.355

Brinkmann, C., Metten, A., Scriba, P., Tagarakis, C.V.M., Wahl, P., Latsch, J., Brixius, K., Bloch, W., 2017. Hypoxia and Hyperoxia Affect Serum Angiogenic Regulators in T2DM Men during Cycling. Int. J. Sports Med. 38, 92–98. https://doi.org/10.1055/s-0042-116823

Broatch, J.R., Petersen, A., Bishop, D.J., 2018. The Influence of Post-Exercise Cold-Water Immersion on Adaptive Responses to Exercise: A Review of the Literature. Sports Med. Auckl. NZ 48, 1369–1387. https://doi.org/10.1007/s40279-018-0910-8

Brocherie, F., Millet, G.P., D'Hulst, G., Van Thienen, R., Deldicque, L., Girard, O., 2018. Repeated maximal-intensity hypoxic exercise superimposed to hypoxic residence boosts skeletal muscle transcriptional responses in elite team-sport athletes. Acta Physiol. 222, e12851. https://doi.org/10.1111/apha.12851

Brodal, P., Ingjer, F., Hermansen, L., 1977. Capillary supply of skeletal muscle fibers in untrained and endurance-trained men. Am. J. Physiol.-Heart Circ. Physiol. https://doi.org/10.1152/ajpheart.1977.232.6.H705

Brody, M.J., Schips, T.G., Vanhoutte, D., Kanisicak, O., Karch, J., Maliken, B.D., Blair, N.S., Sargent, M.A., Prasad, V., Molkentin, J.D., 2016. Dissection of Thrombospondin-4 Domains Involved in Intracellular Adaptive Endoplasmic Reticulum Stress-Responsive Signaling. Mol. Cell. Biol. 36, 2–12. https://doi.org/10.1128/MCB.00607-15

Burtscher, M., Pachinger, O., Ehrenbourg, I., Mitterbauer, G., Faulhaber, M., Pühringer, R., Tkatchouk, E., 2004. Intermittent hypoxia increases exercise tolerance in elderly men with and without coronary artery disease. Int. J. Cardiol. 96, 247–254. https://doi.org/10.1016/j.ijcard.2003.07.021

Camacho-Cardenosa, A., Camacho-Cardenosa, M., Brazo-Sayavera, J., Burtscher, M., Timón, R., Olcina, G., 2018. Effects of High-Intensity Interval Training Under Normobaric Hypoxia on Cardiometabolic Risk Markers in Overweight/Obese Women. High Alt. Med. Biol. 19, 356–366. https://doi.org/10.1089/ham.2018.0059

Camacho-Cardenosa, A., Camacho-Cardenosa, M., Brazo-Sayavera, J., Timón, R., González-Custodio, A., Olcina, G., 2020. Repeated sprint in hypoxia as a time-metabolic efficient strategy to improve physical fitness of obese women. Eur. J. Appl. Physiol. 120, 1051–1061. https://doi.org/10.1007/s00421-020-04344-2

Camacho-Cardenosa, A., Camacho-Cardenosa, M., Olcina, G., Timón, R., Brazo-Sayavera, J., 2019. Detraining effect on overweight/obese women after high-intensity interval training in hypoxia. Scand. J. Med. Sci. Sports 29, 535–543. https://doi.org/10.1111/sms.13380

Carmeliet, P., Jain, R.K., 2011. Molecular mechanisms and clinical applications of angiogenesis. Nature 473, 298–307. https://doi.org/10.1038/nature10144

Casey, D.P., Joyner, M.J., 2012. Compensatory vasodilatation during hypoxic exercise: mechanisms responsible for matching oxygen supply to demand. J. Physiol. 590, 6321–6326. https://doi.org/10.1113/jphysiol.2012.242396

Casey, D.P., Joyner, M.J., 2011. Local control of skeletal muscle blood flow during exercise: influence of available oxygen. J. Appl. Physiol. Bethesda Md 1985 111, 1527–1538. https://doi.org/10.1152/japplphysiol.00895.2011

Castellani, J.W., Young, A.J., 2016. Human physiological responses to cold exposure: Acute responses and acclimatization to prolonged exposure. Auton. Neurosci. 196, 63–74. https://doi.org/10.1016/j.autneu.2016.02.009

Chaillou, T., 2018. Skeletal Muscle Fiber Type in Hypoxia: Adaptation to High-Altitude Exposure and Under Conditions of Pathological Hypoxia. Front. Physiol. 9, 1450. https://doi.org/10.3389/fphys.2018.01450

Chapman, R.F., 2013. The individual response to training and competition at altitude. Br. J. Sports Med. 47 Suppl 1, i40-44. https://doi.org/10.1136/bjsports-2013-092837

Chi, J.-T., Chang, H.Y., Haraldsen, G., Jahnsen, F.L., Troyanskaya, O.G., Chang, D.S., Wang, Z., Rockson, S.G., van de Rijn, M., Botstein, D., Brown, P.O., 2003. Endothelial cell diversity revealed by global expression profiling. Proc. Natl. Acad. Sci. U. S. A. 100, 10623–10628. https://doi.org/10.1073/pnas.1434429100

Chu, L.-Y., Ramakrishnan, D.P., Silverstein, R.L., 2013. Thrombospondin-1 modulates VEGF signaling via CD36 by recruiting SHP-1 to VEGFR2 complex in microvascular endothelial cells. Blood 122, 1822–1832. https://doi.org/10.1182/blood-2013-01-482315

Clarke, R.S.J., Hellon, R.F., Lind, A.R., 1958. The duration of sustained contractions of the human forearm at different muscle temperatures. J. Physiol. 143, 454–473. https://doi.org/10.1113/jphysiol.1958.sp006071

Colburn, T.D., Hirai, D.M., Craig, J.C., Ferguson, S.K., Weber, R.E., Schulze, K.M., Behnke, B.J., Musch, T.I., Poole, D.C., 2020. Transcapillary PO2 gradients in contracting muscles across the fibre type and oxidative continuum. J. Physiol. 598, 3187–3202. https://doi.org/10.1113/JP279608

Conway, E.M., Carmeliet, P., 2004. The diversity of endothelial cells: a challenge for therapeutic angiogenesis. Genome Biol. 5, 207. https://doi.org/10.1186/gb-2004-5-2-207

Coppel, J., Hennis, P., Gilbert-Kawai, E., Grocott, M.P., 2015. The physiological effects of hypobaric hypoxia versus normobaric hypoxia: a systematic review of crossover trials. Extreme Physiol. Med. 4, 2. https://doi.org/10.1186/s13728-014-0021-6

Croley, A.N., Zwetsloot, K.A., Westerkamp, L.M., Ryan, N.A., Pendergast, A.M., Hickner, R.C., Pofahl, W.E., Gavin, T.P., 2005. Lower capillarization, VEGF protein, and VEGF mRNA

response to acute exercise in the vastus lateralis muscle of aged vs. young women. J. Appl. Physiol. Bethesda Md 1985 99, 1872–1879. https://doi.org/10.1152/japplphysiol.00498.2005

Daneshrad, Z., Novel-Chaté, V., Birot, O., Serrurier, B., Sanchez, H., Bigard, A.X., Rossi, A., 2001. Diet restriction plays an important role in the alterations of heart mitochondrial function following exposure of young rats to chronic hypoxia. Pflugers Arch. 442, 12–18. https://doi.org/10.1007/s004240000461

Daneshrad, Z., Verdys, M., Birot, O., Troff, F., Bigard, A.X., Rossi, A., 2003. Chronic hypoxia delays myocardial lactate dehydrogenase maturation in young rats. Exp. Physiol. 88, 405–413. https://doi.org/10.1113/eph8802451

Debevec, T., Millet, G.P., 2014. Discerning normobaric and hypobaric hypoxia: significance of exposure duration. J. Appl. Physiol. Bethesda Md 1985 116, 1255. https://doi.org/10.1152/japplphysiol.00873.2013

Degens, H., Koşar, S.N., Hopman, M.T.E., de Haan, A., 2008. The time course of denervation-induced changes is similar in soleus muscles of adult and old rats. Appl. Physiol. Nutr. Metab. Physiol. Appl. Nutr. Metab. 33, 299–308. https://doi.org/10.1139/H07-189

Degens, H., Turek, Z., Hoofd, L.J., Van't Hof, M.A., Binkhorst, R.A., 1992. The relationship between capillarisation and fibre types during compensatory hypertrophy of the plantaris muscle in the rat. J. Anat. 180 (Pt 3), 455–463.

Delavar, H., Nogueira, L., Wagner, P.D., Hogan, M.C., Metzger, D., Breen, E.C., 2014. Skeletal myofiber VEGF is essential for the exercise training response in adult mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. 306, R586-595. https://doi.org/10.1152/ajpregu.00522.2013

Delezie, J., Handschin, C., 2018. Endocrine Crosstalk Between Skeletal Muscle and the Brain. Front. Neurol. 9, 698. https://doi.org/10.3389/fneur.2018.00698

Desplanches, D., Hoppeler, H., Linossier, M.T., Denis, C., Claassen, H., Dormois, D., Lacour, J.R., Geyssant, A., 1993. Effects of training in normoxia and normobaric hypoxia on human muscle ultrastructure. Pflugers Arch. 425, 263–267. https://doi.org/10.1007/BF00374176

Desplanches, D., Hoppeler, H., Tüscher, L., Mayet, M.H., Spielvogel, H., Ferretti, G., Kayser, B., Leuenberger, M., Grünenfelder, A., Favier, R., 1996. Muscle tissue adaptations of high-altitude natives to training in chronic hypoxia or acute normoxia. J. Appl. Physiol. 81, 1946–1951. https://doi.org/10.1152/jappl.1996.81.5.1946

Deveci, D., Egginton, S., 2003. Cold Exposure Differentially Stimulates Angiogenesis in Glycolytic and Oxidative Muscles of Rats and Hamsters. Exp. Physiol. 88, 741–746. https://doi.org/10.1113/eph8802630

Deveci, D., Egginton, S., 2002. Differing mechanisms of cold-induced changes in capillary supply in m. tibialis anterior of rats and hamsters. J. Exp. Biol. 205, 829–840.

Deveci, D., Marshall, J.M., Egginton, S., 2002. Chronic Hypoxia Induces Prolonged Angiogenesis in Skeletal Muscles of Rat. Exp. Physiol. 87, 287–291. https://doi.org/10.1113/eph8702377

Deveci, D., Marshall, J.M., Egginton, S., 2001. Relationship between capillary angiogenesis, fiber type, and fiber size in chronic systemic hypoxia. Am. J. Physiol.-Heart Circ. Physiol. 281, H241–H252. https://doi.org/10.1152/ajpheart.2001.281.1.H241

D'Hulst, G., Deldicque, L., 2017. Human skeletal muscle wasting in hypoxia: a matter of hypoxic dose? J. Appl. Physiol. Bethesda Md 1985 122, 406–408. https://doi.org/10.1152/japplphysiol.00264.2016

D'Hulst, G., Ferri, A., Naslain, D., Bertrand, L., Horman, S., Francaux, M., Bishop, D.J., Deldicque, L., 2016. Fifteen days of 3,200 m simulated hypoxia marginally regulates markers for protein synthesis and degradation in human skeletal muscle. Hypoxia Auckl. NZ 4, 1–14. https://doi.org/10.2147/HP.S101133

Dinenno, F.A., 2016. Skeletal muscle vasodilation during systemic hypoxia in humans. J. Appl. Physiol. Bethesda Md 1985 120, 216–225. https://doi.org/10.1152/japplphysiol.00256.2015

Ding, H., Liu, Q., Hua, M., Ding, M., Du, H., Zhang, W., Li, Z., Zhang, J., 2012. Associations between Vascular Endothelial Growth Factor Gene Polymorphisms and Susceptibility to Acute Mountain Sickness. J. Int. Med. Res. 40, 2135–2144. https://doi.org/10.1177/030006051204000611

DiPasquale, D.M., Strangman, G.E., Harris, N.S., Muza, S.R., 2016. Acute Mountain Sickness Symptoms Depend on Normobaric versus Hypobaric Hypoxia. BioMed Res. Int. 2016, 6245609. https://doi.org/10.1155/2016/6245609

Domigan, C.K., Warren, C.M., Antanesian, V., Happel, K., Ziyad, S., Lee, S., Krall, A., Duan, L., Torres-Collado, A.X., Castellani, L.W., Elashoff, D., Christofk, H.R., van der Bliek, A.M., Potente, M., Iruela-Arispe, M.L., 2015. Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy. J. Cell Sci. 128, 2236–2248. https://doi.org/10.1242/jcs.163774

Doroudgar, S., Glembotski, C.C., 2013. ATF6 [corrected] and thrombospondin 4: the dynamic duo of the adaptive endoplasmic reticulum stress response. Circ. Res. 112, 9–12. https://doi.org/10.1161/CIRCRESAHA.112.280560

Dorward, D.A., Roger Thompson, A.A., Kenneth Baillie, J., MacDougall, M., Hirani, N., 2007. Change in plasma vascular endothelial growth factor during onset and recovery from acute mountain sickness. Respir. Med. 101, 587–594. https://doi.org/10.1016/j.rmed.2006.06.014

D'Souza, R.F., Zeng, N., Markworth, J.F., Figueiredo, V.C., Roberts, L.A., Raastad, T., Coombes, J.S., Peake, J.M., Cameron-Smith, D., Mitchell, C.J., 2018. Divergent effects of cold water immersion versus active recovery on skeletal muscle fiber type and angiogenesis in young men. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 314, R824–R833. https://doi.org/10.1152/ajpregu.00421.2017

Duchamp, C., Cohen-Adad, F., Rouanet, J.L., Barré, H., 1992. Histochemical arguments for muscular non-shivering thermogenesis in muscovy ducklings. J. Physiol. 457, 27–45. https://doi.org/10.1113/jphysiol.1992.sp019363

Dunford, E.C., Leclair, E., Aiken, J., Mandel, E.R., Haas, T.L., Birot, O., Riddell, M.C., 2017. The effects of voluntary exercise and prazosin on capillary rarefaction and metabolism in streptozotocin-induced diabetic male rats. J. Appl. Physiol. Bethesda Md 1985 122, 492–502. https://doi.org/10.1152/japplphysiol.00762.2016

Egginton, S., 2011. In vivo shear stress response. Biochem. Soc. Trans. 39, 1633–1638. https://doi.org/10.1042/BST20110715

Egginton, S., 2009. Invited review: activity-induced angiogenesis. Pflüg. Arch. - Eur. J. Physiol. 457, 963–977. https://doi.org/10.1007/s00424-008-0563-9

Egginton, S., 2002. Temperature and angiogenesis: the possible role of mechanical factors in capillary growth. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 132, 773–787. https://doi.org/10.1016/S1095-6433(02)00047-8

Egginton, S., Birot, O., 2014. Angiogenesis: growth points. Microcirc. N. Y. N 1994 21, 276–277. https://doi.org/10.1111/micc.12131

Egginton, S., Fairney, J., Bratcher, J., 2001. Differential Effects of Cold Exposure on Muscle Fibre Composition and Capillary Supply in Hibernator and Non-Hibernator Rodents. Exp. Physiol. 86, 629–639. https://doi.org/10.1113/eph8602260

Egginton, S., Hudlická, O., Brown, M.D., Walter, H., Weiss, J.B., Bate, A., 1998. Capillary growth in relation to blood flow and performance in overloaded rat skeletal muscle. J. Appl. Physiol. Bethesda Md 1985 85, 2025–2032. https://doi.org/10.1152/jappl.1998.85.6.2025

Espinoza, J.R., Alvarez, G., León-Velarde, F., Ju Preciado, H.F., Macarlupu, J.-L., Rivera-Ch, M., Rodriguez, J., Favier, J., Gimenez-Roqueplo, A.-P., Richalet, J.-P., 2014. Vascular Endothelial Growth Factor-A Is Associated with Chronic Mountain Sickness in the Andean Population. High Alt. Med. Biol. 15, 146–154. https://doi.org/10.1089/ham.2013.1121

Evers-van Gogh, I.J.A., Alex, S., Stienstra, R., Brenkman, A.B., Kersten, S., Kalkhoven, E., 2015. Electric Pulse Stimulation of Myotubes as an In Vitro Exercise Model: Cell-Mediated and Non-Cell-Mediated Effects. Sci. Rep. 5, 10944. https://doi.org/10.1038/srep10944

Ewan, L.C., Jopling, H.M., Jia, H., Mittar, S., Bagherzadeh, A., Howell, G.J., Walker, J.H., Zachary, I.C., Ponnambalam, S., 2006. Intrinsic Tyrosine Kinase Activity is Required for Vascular Endothelial Growth Factor Receptor 2 Ubiquitination, Sorting and Degradation in Endothelial Cells. Traffic 7, 1270–1282. https://doi.org/10.1111/j.1600-0854.2006.00462.x

Fabel, Klaus, Fabel, Konstanze, Tam, B., Kaufer, D., Baiker, A., Simmons, N., Kuo, C.J., Palmer, T.D., 2003. VEGF is necessary for exercise-induced adult hippocampal neurogenesis. Eur. J. Neurosci. 18, 2803–2812. https://doi.org/10.1111/j.1460-9568.2003.03041.x

Faiss, R., Girard, O., Millet, G.P., 2013. Advancing hypoxic training in team sports: from intermittent hypoxic training to repeated sprint training in hypoxia: Table 1. Br. J. Sports Med. 47, i45–i50. https://doi.org/10.1136/bjsports-2013-092741

Favier, F.B., Britto, F.A., Freyssenet, D.G., Bigard, X.A., Benoit, H., 2015. HIF-1-driven skeletal muscle adaptations to chronic hypoxia: molecular insights into muscle physiology. Cell. Mol. Life Sci. 72, 4681–4696. https://doi.org/10.1007/s00018-015-2025-9

Ferrara, N., Houck, K.A., Jakeman, L.B., Winer, J., Leung, D.W., 1991. The vascular endothelial growth factor family of polypeptides. J. Cell. Biochem. 47, 211–218. https://doi.org/10.1002/jcb.240470305

Fraisl, P., Mazzone, M., Schmidt, T., Carmeliet, P., 2009. Regulation of Angiogenesis by Oxygen and Metabolism. Dev. Cell 16, 167–179. https://doi.org/10.1016/j.devcel.2009.01.003

Frontera, W.R., Ochala, J., 2015. Skeletal Muscle: A Brief Review of Structure and Function. Calcif. Tissue Int. 96, 183–195. https://doi.org/10.1007/s00223-014-9915-y

Fujino, H., Kohzuki, H., Takeda, I., Kiyooka, T., Miyasaka, T., Mohri, S., Shimizu, J., Kajiya, F., 2005. Regression of capillary network in atrophied soleus muscle induced by hindlimb unweighting. J. Appl. Physiol. Bethesda Md 1985 98, 1407–1413. https://doi.org/10.1152/japplphysiol.00961.2004

Fujita, J., 1999. Cold shock response in mammalian cells. J. Mol. Microbiol. Biotechnol. 1, 243–255.

Fulbert, C., Gaude, C., Sulpice, E., Chabardès, S., Ratel, D., 2019. Moderate hypothermia inhibits both proliferation and migration of human glioblastoma cells. J. Neurooncol. 144, 489–499. https://doi.org/10.1007/s11060-019-03263-3

Fuller-Jackson, J.-P., Henry, B.A., 2018. Adipose and skeletal muscle thermogenesis: studies from large animals. J. Endocrinol. 237, R99–R115. https://doi.org/10.1530/JOE-18-0090

Gardner, A.W., Poehlman, E.T., 1995. Exercise rehabilitation programs for the treatment of claudication pain. A meta-analysis. JAMA 274, 975–980.

Gavin, T.P., 2009. Basal and exercise-induced regulation of skeletal muscle capillarization. Exerc. Sport Sci. Rev. 37, 86–92. https://doi.org/10.1097/JES.0b013e31819c2e9b

Gavin, T.P., Drew, J.L., Kubik, C.J., Pofahl, W.E., Hickner, R.C., 2007. Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. Acta Physiol. 191, 139–146. https://doi.org/10.1111/j.1748-1716.2007.01723.x

Gavin, T.P., Wagner, P.D., 2001. Effect of short-term exercise training on angiogenic growth factor gene responses in rats. J. Appl. Physiol. Bethesda Md 1985 90, 1219–1226. https://doi.org/10.1152/jappl.2001.90.4.1219

Gavin, T.P., Westerkamp, L.M., Zwetsloot, K.A., 2006. Soleus, plantaris and gastrocnemius VEGF mRNA responses to hypoxia and exercise are preserved in aged compared with young

female C57BL/6 mice. Acta Physiol. 188, 113–121. https://doi.org/10.1111/j.1748-1716.2006.01609.x

Gerber, H.P., Condorelli, F., Park, J., Ferrara, N., 1997. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J. Biol. Chem. 272, 23659–23667. https://doi.org/10.1074/jbc.272.38.23659

Gilbert-Kawai, E.T., Milledge, J.S., Grocott, M.P.W., Martin, D.S., 2014. King of the Mountains: Tibetan and Sherpa Physiological Adaptations for Life at High Altitude. Physiology 29, 388–402. https://doi.org/10.1152/physiol.00018.2014

Girard, O., Amann, M., Aughey, R., Billaut, F., Bishop, D.J., Bourdon, P., Buchheit, M., Chapman, R., D'Hooghe, M., Garvican-Lewis, L.A., Gore, C.J., Millet, G.P., Roach, G.D., Sargent, C., Saunders, P.U., Schmidt, W., Schumacher, Y.O., 2013. Position statement—altitude training for improving team-sport players' performance: current knowledge and unresolved issues. Br. J. Sports Med. 47, i8–i16. https://doi.org/10.1136/bjsports-2013-093109

Girard, O., Brocherie, F., Goods, P.S.R., Millet, G.P., 2020. An Updated Panorama of "Living Low-Training High" Altitude/Hypoxic Methods. Front. Sports Act. Living 2, 26. https://doi.org/10.3389/fspor.2020.00026

Girard, O., Brocherie, F., Millet, G.P., 2017. Effects of Altitude/Hypoxia on Single- and Multiple-Sprint Performance: A Comprehensive Review. Sports Med. Auckl. NZ 47, 1931–1949. https://doi.org/10.1007/s40279-017-0733-z

Girard, O., Chalabi, H., 2013. Could altitude training benefit team-sport athletes? Br. J. Sports Med. 47, i4–i5. https://doi.org/10.1136/bjsports-2013-092807

Girard, O., Koehle, M.S., MacInnis, M.J., Guenette, J.A., Koehle, M.S., Verges, S., Rupp, T., Jubeau, M., Perrey, S., Millet, G.Y., Chapman, R.F., Levine, B.D., Conkin, J., Wessel, J.H., Nespoulet, H., Wuyam, B., Tamisier, R., Verges, S., Levy, P., Casey, D.P., Taylor, B.J., Snyder, E.M., Johnson, B.D., Laymon, A.S., Stickford, J.L., Weavil, J.C., Loeppky, J.A., Pun, M., Schommer, K., Bartsch, P., Vagula, M.C., Nelatury, C.F., 2012. Comments on Point:Counterpoint: Hypobaric hypoxia induces/does not induce different responses from normobaric hypoxia. J. Appl. Physiol. Bethesda Md 1985 112, 1788–1794. https://doi.org/10.1152/japplphysiol.00356.2012

Girard, O., Pluim, B.M., 2013. Improving team-sport player's physical performance with altitude training: from beliefs to scientific evidence. Br. J. Sports Med. 47, i2–i3. https://doi.org/10.1136/bjsports-2013-093119

Gliemann, L., Buess, R., Nyberg, M., Hoppeler, H., Odriozola, A., Thaning, P., Hellsten, Y., Baum, O., Mortensen, S.P., 2015. Capillary growth, ultrastructure remodelling and exercise training in skeletal muscle of essential hypertensive patients. Acta Physiol. Oxf. Engl. 214, 210–220. https://doi.org/10.1111/apha.12501

Gomarasca, M., Banfi, G., Lombardi, G., 2020. Myokines: The endocrine coupling of skeletal muscle and bone. Adv. Clin. Chem. 94, 155–218. https://doi.org/10.1016/bs.acc.2019.07.010

Gouzi, F., Préfaut, C., Abdellaoui, A., Roudier, E., de Rigal, P., Molinari, N., Laoudj-Chenivesse, D., Mercier, J., Birot, O., Hayot, M., 2013. Blunted muscle angiogenic training-response in COPD patients versus sedentary controls. Eur. Respir. J. 41, 806–814. https://doi.org/10.1183/09031936.00053512

Green, H.J., Sutton, J.R., Cymerman, A., Young, P.M., Houston, C.S., 1989. Operation Everest II: adaptations in human skeletal muscle. J. Appl. Physiol. 66, 2454–2461. https://doi.org/10.1152/jappl.1989.66.5.2454

Green, H.J., Sutton, J.R., Wolfel, E.E., Reeves, J.T., Butterfield, G.E., Brooks, G.A., 1992. Altitude acclimatization and energy metabolic adaptations in skeletal muscle during exercise. J. Appl. Physiol. 73, 2701–2708. https://doi.org/10.1152/jappl.1992.73.6.2701

Greenaway, J., Lawler, J., Moorehead, R., Bornstein, P., Lamarre, J., Petrik, J., 2007. Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1). J. Cell. Physiol. 210, 807–818. https://doi.org/10.1002/jcp.20904

Gunga, H.-C., Fries, D., Humpeler, E., Kirsch, K., Boldt, L.-E., Koralewski, E., Johannes, B., Klingler, A., Mittermayr, M., Röcker, L., Yaban, B., Behn, C., Jelkmann, W., Schobersberger, W., 2003. Austrian Moderate Altitude Study (AMAS 2000) – fluid shifts, erythropoiesis, and angiogenesis in patients with metabolic syndrome at moderate altitude (≅1700 m). Eur. J. Appl. Physiol. 88, 497–505. https://doi.org/10.1007/s00421-002-0734-x

Gunga, H.-C., Kirsch, K., R[illegible]cker, L., Behn, C., Koralewski, E., Davila, E.H., Estrada, M.I., Johannes, B., Wittels, P., Jelkmann, W., 1999. Vascular endothelial growth factor in exercising humans under different environmental conditions. Eur. J. Appl. Physiol. 79, 484–490. https://doi.org/10.1007/s004210050541

Gupta, K., Gupta, P., Wild, R., Ramakrishnan, S., Hebbel, R.P., 1999. Binding and displacement of vascular endothelial growth factor (VEGF) by thrombospondin: Effect on human microvascular endothelial cell proliferation and angiogenesis. Angiogenesis 3, 147–158. https://doi.org/10.1023/A:1009018702832

Gustafsson, T., Ameln, H., Fischer, H., Sundberg, C.J., Timmons, J.A., Jansson, E., 2005. VEGF-A splice variants and related receptor expression in human skeletal muscle following submaximal exercise. J. Appl. Physiol. Bethesda Md 1985 98, 2137–2146. https://doi.org/10.1152/japplphysiol.01402.2004

Gustafsson, T., Knutsson, A., Puntschart, A., Kaijser, L., Nordqvist, A.-C.S., Sundberg, C.J., Jansson, E., 2002. Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. Pflugers Arch. 444, 752–759. https://doi.org/10.1007/s00424-002-0845-6

Gustafsson, T., Kraus, W.E., 2001. Exercise-induced angiogenesis-related growth and transcription factors in skeletal muscle, and their modification in muscle pathology. Front. Biosci. J. Virtual Libr. 6, D75-89. https://doi.org/10.2741/gustafss

Haas, T.L., Nwadozi, E., 2015. Regulation of skeletal muscle capillary growth in exercise and disease. Appl. Physiol. Nutr. Metab. Physiol. Appl. Nutr. Metab. 40, 1221–1232. https://doi.org/10.1139/apnm-2015-0336

Hanaoka, M., Droma, Y., Naramoto, A., Honda, T., Kobayashi, T., Kubo, K., 2003. Vascular endothelial growth factor in patients with high-altitude pulmonary edema. J. Appl. Physiol. 94, 1836–1840. https://doi.org/10.1152/japplphysiol.00575.2002

Hellsten, Y., Hoier, B., 2014. Capillary growth in human skeletal muscle: physiological factors and the balance between pro-angiogenic and angiostatic factors. Biochem. Soc. Trans. 42, 1616–1622. https://doi.org/10.1042/BST20140197

Hepple, R.T., 2000. Skeletal muscle: microcirculatory adaptation to metabolic demand. Med. Sci. Sports Exerc. 32, 117–123. https://doi.org/10.1097/00005768-200001000-00018

Hepple, R.T., Agey, P.J., Hazelwood, L., Szewczak, J.M., MacMillen, R.E., Mathieu-Costello, O., 1998. Increased capillarity in leg muscle of finches living at altitude. J. Appl. Physiol. Bethesda Md 1985 85, 1871–1876. https://doi.org/10.1152/jappl.1998.85.5.1871

Heroux, O., St. Pierre, J., 1956. Effect of Cold Acclimation on Vascularization of Ears, Heart, Liver and Muscles of White Rats. Am. J. Physiol.-Leg. Content 188, 163–168. https://doi.org/10.1152/ajplegacy.1956.188.1.163

Herpin, P., Lossec, G., Schmidt, I., Cohen-Adad, F., Duchamp, C., Lefaucheur, L., Goglia, F., Lanni, A., 2002. Effect of age and cold exposure on morphofunctional characteristics of skeletal muscle in neonatal pigs. Pfl�gers Arch. Eur. J. Physiol. 444, 610–618. https://doi.org/10.1007/s00424-002-0867-0

Hirai, D.M., Colburn, T.D., Craig, J.C., Hotta, K., Kano, Y., Musch, T.I., Poole, D.C., 2019. Skeletal muscle interstitial O2 pressures: bridging the gap between the capillary and myocyte. Microcirc. N. Y. N 1994 26, e12497. https://doi.org/10.1111/micc.12497

Hirai, D.M., Craig, J.C., Colburn, T.D., Eshima, H., Kano, Y., Sexton, W.L., Musch, T.I., Poole, D.C., 2018. Skeletal muscle microvascular and interstitial PO2 from rest to contractions. J. Physiol. 596, 869–883. https://doi.org/10.1113/JP275170

Hoffmann, C., Weigert, C., 2017. Skeletal Muscle as an Endocrine Organ: The Role of Myokines in Exercise Adaptations. Cold Spring Harb. Perspect. Med. 7, a029793. https://doi.org/10.1101/cshperspect.a029793

Hohberg, M., Knöchel, J., Hoffmann, C.J., Chlench, S., Wunderlich, W., Alter, A., Maroski, J., Vorderwülbecke, B.J., Da Silva-Azevedo, L., Knudsen, R., Lehmann, R., Fiedorowicz, K., Bongrazio, M., Nitsche, B., Hoepfner, M., Styp-Rekowska, B., Pries, A.R., Zakrzewicz, A.,

2011. Expression of ADAMTS1 in endothelial cells is induced by shear stress and suppressed in sprouting capillaries. J. Cell. Physiol. 226, 350–361. https://doi.org/10.1002/jcp.22340

Hoier, B., Hellsten, Y., 2014. Exercise-Induced Capillary Growth in Human Skeletal Muscle and the Dynamics of VEGF. Microcirculation 21, 301–314. https://doi.org/10.1111/micc.12117

Hoier, B., Nordsborg, N., Andersen, S., Jensen, L., Nybo, L., Bangsbo, J., Hellsten, Y., 2012. Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training: Angiogenic factors in human muscle. J. Physiol. 590, 595–606. https://doi.org/10.1113/jphysiol.2011.216135

Hoier, B., Olsen, K., Hanskov, D.J.A., Jorgensen, M., Norup, L.R., Hellsten, Y., 2020. Early time course of change in angiogenic proteins in human skeletal muscle and vascular cells with endurance training. Scand. J. Med. Sci. Sports 30, 1117–1131. https://doi.org/10.1111/sms.13665

Hoppeler, H., 1999. Vascular growth in hypoxic skeletal muscle. Adv. Exp. Med. Biol. 474, 277–286. https://doi.org/10.1007/978-1-4615-4711-2_21

Hoppeler, H., Desplanches, D., 1992. Muscle structural modifications in hypoxia. Int. J. Sports Med. 13 Suppl 1, S166-168. https://doi.org/10.1055/s-2007-1024628

Hoppeler, H., Howald, H., Cerretelli, P., 1990a. Human muscle structure after exposure to extreme altitude. Experientia 46, 1185–1187. https://doi.org/10.1007/BF01936933

Hoppeler, H., Hudlicka, O., Uhlmann, E., 1987. Relationship between mitochondria and oxygen consumption in isolated cat muscles. J. Physiol. 385, 661–675. https://doi.org/10.1113/jphysiol.1987.sp016513

Hoppeler, H., Kayar, S., 1988. Capillarity and Oxidative Capacity of Muscles. Physiology 3, 113–116. https://doi.org/10.1152/physiologyonline.1988.3.3.113

Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S.R., Cerretelli, P., 1990b. Morphological adaptations of human skeletal muscle to chronic hypoxia. Int. J. Sports Med. 11 Suppl 1, S3-9. https://doi.org/10.1055/s-2007-1024846

Hoppeler, H., Lüthi, P., Claassen, H., Weibel, E.R., Howald, H., 1973. The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. Pflugers Arch. 344, 217–232. https://doi.org/10.1007/BF00588462

Hoppeler, H., Vogt, M., 2001. Muscle tissue adaptations to hypoxia. J. Exp. Biol. 204, 3133–3139.

Hoppeler, H., Vogt, M., Weibel, E.R., Flück, M., 2003. Response of skeletal muscle mitochondria to hypoxia. Exp. Physiol. 88, 109–119. https://doi.org/10.1113/eph8802513

Horscroft, J.A., Kotwica, A.O., Laner, V., West, J.A., Hennis, P.J., Levett, D.Z.H., Howard, D.J., Fernandez, B.O., Burgess, S.L., Ament, Z., Gilbert-Kawai, E.T., Vercueil, A., Landis, B.D., Mitchell, K., Mythen, M.G., Branco, C., Johnson, R.S., Feelisch, M., Montgomery, H.E., Griffin, J.L., Grocott, M.P.W., Gnaiger, E., Martin, D.S., Murray, A.J., 2017. Metabolic basis to Sherpa

altitude adaptation. Proc. Natl. Acad. Sci. 114, 6382–6387. https://doi.org/10.1073/pnas.1700527114

Horscroft, J.A., Murray, A.J., 2014. Skeletal muscle energy metabolism in environmental hypoxia: climbing towards consensus. Extreme Physiol. Med. 3, 19. https://doi.org/10.1186/2046-7648-3-19

Houck, K.A., Ferrara, N., Winer, J., Cachianes, G., Li, B., Leung, D.W., 1991. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol. Endocrinol. Baltim. Md 5, 1806–1814. https://doi.org/10.1210/mend-5-12-1806

Howlett, R.A., Gonzalez, N.C., Wagner, H.E., Fu, Z., Britton, S.L., Koch, L.G., Wagner, P.D., 2003. Selected Contribution: Skeletal muscle capillarity and enzyme activity in rats selectively bred for running endurance. J. Appl. Physiol. 94, 1682–1688. https://doi.org/10.1152/japplphysiol.00556.2002

Hudlicka, O., 2011. Microcirculation in skeletal muscle. Muscles Ligaments Tendons J. 1, 3–11.

Hudlicka, O., Brown, M., Egginton, S., 1992. Angiogenesis in skeletal and cardiac muscle. Physiol. Rev. 72, 369–417. https://doi.org/10.1152/physrev.1992.72.2.369

Hudlicka, O., Brown, M.D., 2009. Adaptation of skeletal muscle microvasculature to increased or decreased blood flow: role of shear stress, nitric oxide and vascular endothelial growth factor. J. Vasc. Res. 46, 504–512. https://doi.org/10.1159/000226127

Hudlicka, O., Brown, M.D., Egginton, S., Dawson, J.M., 1994. Effect of long-term electrical stimulation on vascular supply and fatigue in chronically ischemic muscles. J. Appl. Physiol. Bethesda Md 1985 77, 1317–1324. https://doi.org/10.1152/jappl.1994.77.3.1317

Hudlicka, O., Hoppeler, H., Uhlmann, E., 1987. Relationship between the size of the capillary bed and oxidative capacity in various cat skeletal muscles. Pflugers Arch. 410, 369–375. https://doi.org/10.1007/BF00586513

Huez, I., Créancier, L., Audigier, S., Gensac, M.C., Prats, A.C., Prats, H., 1998. Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. Mol. Cell. Biol. 18, 6178–6190. https://doi.org/10.1128/MCB.18.11.6178

Hutter, J., Habler, O., Kleen, M., Tiede, M., Podtschaske, A., Kemming, G., Corso, C., Batra, S., Keipert, P., Faithfull, S., Messmer, K., 1999. Effect of acute normovolemic hemodilution on distribution of blood flow and tissue oxygenation in dog skeletal muscle. J. Appl. Physiol. 86, 860–866. https://doi.org/10.1152/jappl.1999.86.3.860

Ihsan, M., Abbiss, C.R., Allan, R., 2021. Adaptations to Post-exercise Cold Water Immersion: Friend, Foe, or Futile? Front. Sports Act. Living 3, 201. https://doi.org/10.3389/fspor.2021.714148

Ihsan, M., Watson, G., Choo, H.C., Lewandowski, P., Papazzo, A., Cameron-Smith, D., Abbiss, C.R., 2014. Postexercise Muscle Cooling Enhances Gene Expression of PGC-1α. Med. Sci. Sports Exerc. 46, 1900–1907. https://doi.org/10.1249/MSS.0000000000000308

Iruela-Arispe, M.L., Carpizo, D., Luque, A., 2003. ADAMTS1: a matrix metalloprotease with angioinhibitory properties. Ann. N. Y. Acad. Sci. 995, 183–190. https://doi.org/10.1111/j.1749-6632.2003.tb03221.x

Ishii, H., Oota, I., Arakawa, T., Takuma, T., 2002. Differential gene expression of vascular endothelial growth factor isoforms and their receptors in the development of the rat masseter muscle. Arch. Oral Biol. 47, 505–510. https://doi.org/10.1016/S0003-9969(02)00033-X

Jackson, C.G., Sillau, A.H., Banchero, N., 1987. Fiber composition and capillarity in growing guinea pigs acclimated to cold and cold plus hypoxia. Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. N. Y. N 185, 101–106. https://doi.org/10.3181/00379727-185-42524

Jensen, L., Bangsbo, J., Hellsten, Y., 2004. Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. J. Physiol. 557, 571–582. https://doi.org/10.1113/jphysiol.2003.057711

Jiménez, B., Volpert, O.V., Crawford, S.E., Febbraio, M., Silverstein, R.L., Bouck, N., 2000. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat. Med. 6, 41–48. https://doi.org/10.1038/71517

Johnson, P.C., Vandegriff, K., Tsai, A.G., Intaglietta, M., 2005. Effect of acute hypoxia on microcirculatory and tissue oxygen levels in rat cremaster muscle. J. Appl. Physiol. Bethesda Md 1985 98, 1177–1184. https://doi.org/10.1152/japplphysiol.00591.2004

Joo, C.H., Allan, R., Drust, B., Close, G.L., Jeong, T.S., Bartlett, J.D., Mawhinney, C., Louhelainen, J., Morton, J.P., Gregson, W., 2016. Passive and post-exercise cold-water immersion augments PGC-1α and VEGF expression in human skeletal muscle. Eur. J. Appl. Physiol. 116, 2315–2326. https://doi.org/10.1007/s00421-016-3480-1

Joyner, M.J., Casey, D.P., 2014. Muscle blood flow, hypoxia, and hypoperfusion. J. Appl. Physiol. Bethesda Md 1985 116, 852–857. https://doi.org/10.1152/japplphysiol.00620.2013

Jung, W.-S., Kim, S.-W., Kim, J.-W., Park, H.-Y., 2021. Resistance Training in Hypoxia as a New Therapeutic Modality for Sarcopenia—A Narrative Review. Life 11, 106. https://doi.org/10.3390/life11020106

Kaiser, R., Frantz, C., Bals, R., Wilkens, H., 2016. The role of circulating thrombospondin-1 in patients with precapillary pulmonary hypertension. Respir. Res. 17, 96. https://doi.org/10.1186/s12931-016-0412-x

Kasai, N., Kojima, C., Sumi, D., Ikutomo, A., Goto, K., 2019. Inflammatory, Oxidative Stress, and Angiogenic Growth Factor Responses to Repeated-Sprint Exercise in Hypoxia. Front. Physiol. 10, 844. https://doi.org/10.3389/fphys.2019.00844

Kasperska, A., Zembron-Lacny, A., 2020. The effect of intermittent hypoxic exposure on erythropoietic response and hematological variables in elite athletes. Physiol. Res. 283–290. https://doi.org/10.33549/physiolres.934316

Kaur, S., Martin-Manso, G., Pendrak, M.L., Garfield, S.H., Isenberg, J.S., Roberts, D.D., 2010. Thrombospondin-1 Inhibits VEGF Receptor-2 Signaling by Disrupting Its Association with CD47. J. Biol. Chem. 285, 38923–38932. https://doi.org/10.1074/jbc.M110.172304

Kayser, B., Hoppeler, H., Claassen, H., Cerretelli, P., 1991. Muscle structure and performance capacity of Himalayan Sherpas. J. Appl. Physiol. 70, 1938–1942. https://doi.org/10.1152/jappl.1991.70.5.1938

Kayser, B., Hoppeler, H., Desplanches, D., Marconi, C., Broers, B., Cerretelli, P., 1996. Muscle ultrastructure and biochemistry of lowland Tibetans. J. Appl. Physiol. 81, 419–425. https://doi.org/10.1152/jappl.1996.81.1.419

Ke, Q., Costa, M., 2006. Hypoxia-inducible factor-1 (HIF-1). Mol. Pharmacol. 70, 1469–1480. https://doi.org/10.1124/mol.106.027029

Kiley, J.P., Eldridge, F.L., Millhorn, D.E., 1984. Brain, blood and rectal temperature during whole body cooling. Comp. Biochem. Physiol. A Physiol. 79, 631–634. https://doi.org/10.1016/0300-9629(84)90460-2

Kim, J.C., Yi, H.K., Hwang, P.H., Yoon, J.S., Kim, H.J., Kawano, F., Ohira, Y., Kim, C.K., 2005. Effects of cold-water immersion on VEGF mRNA and protein expression in heart and skeletal muscles of rats. Acta Physiol. Scand. 183, 389–397. https://doi.org/10.1111/j.1365-201X.2005.01415.x

Kim, S., Choi, J.-Y., Moon, S., Park, D.-H., Kwak, H.-B., Kang, J.-H., 2019. Roles of myokines in exercise-induced improvement of neuropsychiatric function. Pflugers Arch. 471, 491–505. https://doi.org/10.1007/s00424-019-02253-8

Kindig, C.A., Howlett, R.A., Hogan, M.C., 2003. Effect of extracellular P O_2 on the fall in intracellular P O_2 in contracting single myocytes. J. Appl. Physiol. 94, 1964–1970. https://doi.org/10.1152/japplphysiol.00893.2002

Kishlyansky, M., Vojnovic, J., Roudier, E., Gineste, C., Decary, S., Forn, P., Bergeron, R., Desplanches, D., Birot, O., 2010. Striated muscle angio-adaptation requires changes in Vasohibin-1 expression pattern. Biochem. Biophys. Res. Commun. 399, 359–364. https://doi.org/10.1016/j.bbrc.2010.07.076

Kissane, R.W.P., Al-Shammari, A.A., Egginton, S., 2021. The importance of capillary distribution in supporting muscle function, building on Krogh's seminal ideas. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 254, 110889. https://doi.org/10.1016/j.cbpa.2020.110889

Kivelä, R., Silvennoinen, M., Lehti, M., Jalava, S., Vihko, V., Kainulainen, H., 2008. Exercise-induced expression of angiogenic growth factors in skeletal muscle and in capillaries of healthy and diabetic mice. Cardiovasc. Diabetol. 7, 13. https://doi.org/10.1186/1475-2840-7-13

Kivelä, R., Silvennoinen, M., Touvra, A.-M., Lehti, T.M., Kainulainen, H., Vihko, V., 2006. Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 20, 1570–1572. https://doi.org/10.1096/fj.05-4780fje

Knapp, A.E., Goldberg, D., Delavar, H., Trisko, B.M., Tang, K., Hogan, M.C., Wagner, P.D., Breen, E.C., 2016. Skeletal myofiber VEGF regulates contraction-induced perfusion and exercise capacity but not muscle capillarity in adult mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. 311, R192-199. https://doi.org/10.1152/ajpregu.00533.2015

Koester-Hegmann, C., Bengoetxea, H., Kosenkov, D., Thiersch, M., Haider, T., Gassmann, M., Schneider Gasser, E.M., 2019. High-Altitude Cognitive Impairment Is Prevented by Enriched Environment Including Exercise via VEGF Signaling. Front. Cell. Neurosci. 12, 532. https://doi.org/10.3389/fncel.2018.00532

Kong, Z., Shi, Q., Nie, J., Tong, T.K., Song, L., Yi, L., Hu, Y., 2017. High-Intensity Interval Training in Normobaric Hypoxia Improves Cardiorespiratory Fitness in Overweight Chinese Young Women. Front. Physiol. 8, 175. https://doi.org/10.3389/fphys.2017.00175

Kosmidou, I., Xagorari, A., Roussos, C., Papapetropoulos, A., 2001. Reactive oxygen species stimulate VEGF production from C(2)C(12) skeletal myotubes through a PI3K/Akt pathway. Am. J. Physiol. Lung Cell. Mol. Physiol. 280, L585-592. https://doi.org/10.1152/ajplung.2001.280.4.L585

Krapf, S., Schjølberg, T., Asoawe, L., Honkanen, S.K., Kase, E.T., Thoresen, G.H., Haugen, F., 2021. Novel methods for cold exposure of skeletal muscle in vivo and in vitro show temperature-dependent myokine production. J. Therm. Biol. 98, 102930. https://doi.org/10.1016/j.jtherbio.2021.102930

Kraus, R.M., Stallings, H.W., Yeager, R.C., Gavin, T.P., 2004. Circulating plasma VEGF response to exercise in sedentary and endurance-trained men. J. Appl. Physiol. 96, 1445–1450. https://doi.org/10.1152/japplphysiol.01031.2003

Krogh, A., 1919a. The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. J. Physiol. 52, 391–408. https://doi.org/10.1113/jphysiol.1919.sp001838

Krogh, A., 1919b. The number and distribution of capillaries in muscles with calculations of the oxygen pressure head necessary for supplying the tissue. J. Physiol. 52, 409–415. https://doi.org/10.1113/jphysiol.1919.sp001839

Krogh, A., 1919c. The supply of oxygen to the tissues and the regulation of the capillary circulation. J. Physiol. 52, 457–474. https://doi.org/10.1113/jphysiol.1919.sp001844

Lam, B., Nwadozi, E., Haas, T.L., Birot, O., Roudier, E., 2021. High Glucose Treatment Limits Drosha Protein Expression and Alters AngiomiR Maturation in Microvascular Primary Endothelial Cells via an Mdm2-dependent Mechanism. Cells 10, 742. https://doi.org/10.3390/cells10040742

Lampugnani, M.G., Orsenigo, F., Gagliani, M.C., Tacchetti, C., Dejana, E., 2006. Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. J. Cell Biol. 174, 593–604. https://doi.org/10.1083/jcb.200602080

Lawler, P.R., Lawler, J., 2012. Molecular Basis for the Regulation of Angiogenesis by Thrombospondin-1 and -2. Cold Spring Harb. Perspect. Med. 2, a006627. https://doi.org/10.1101/cshperspect.a006627

Lee, S., Chen, T.T., Barber, C.L., Jordan, M.C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K.P., Iruela-Arispe, M.L., 2007. Autocrine VEGF signaling is required for vascular homeostasis. Cell 130, 691–703. https://doi.org/10.1016/j.cell.2007.06.054

Leick, L., Hellsten, Y., Fentz, J., Lyngby, S.S., Wojtaszewski, J.F.P., Hidalgo, J., Pilegaard, H., 2009. PGC-1alpha mediates exercise-induced skeletal muscle VEGF expression in mice. Am. J. Physiol. Endocrinol. Metab. 297, E92-103. https://doi.org/10.1152/ajpendo.00076.2009

Lemieux, P., Birot, O., 2021. Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story. Front. Physiol. 12, 735557. https://doi.org/10.3389/fphys.2021.735557

Levett, D.Z., Radford, E.J., Menassa, D.A., Graber, E.F., Morash, A.J., Hoppeler, H., Clarke, K., Martin, D.S., Ferguson-Smith, A.C., Montgomery, H.E., Grocott, M.P.W., Murray, A.J., the Caudwell Xtreme Everest Research Group, 2012. Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. FASEB J. 26, 1431–1441. https://doi.org/10.1096/fj.11-197772

Levine, B.D., 2002. Intermittent hypoxic training: fact and fancy. High Alt. Med. Biol. 3, 177–193. https://doi.org/10.1089/15270290260131911

Levine, B.D., Stray-Gundersen, J., 2006. Dose-Response of Altitude Training: How Much Altitude is Enough?, in: Roach, R.C., Wagner, P.D., Hackett, P.H. (Eds.), Hypoxia and Exercise. Springer US, Boston, MA, pp. 233–247. https://doi.org/10.1007/978-0-387-34817-9_20

Lindholm, M.E., Fischer, H., Poellinger, L., Johnson, R.S., Gustafsson, T., Sundberg, C.J., Rundqvist, H., 2014. Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. Am. J. Physiol. Regul. Integr. Comp. Physiol. 307, R248-255. https://doi.org/10.1152/ajpregu.00036.2013

Lindholm, M.E., Rundqvist, H., 2016. Skeletal muscle hypoxia-inducible factor-1 and exercise. Exp. Physiol. 101, 28–32. https://doi.org/10.1113/EP085318

Little, A.G., Seebacher, F., 2016. Thermal conditions experienced during differentiation affect metabolic and contractile phenotypes of mouse myotubes. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 311, R457–R465. https://doi.org/10.1152/ajpregu.00148.2016

Liu, G., Mac Gabhann, F., Popel, A.S., 2012. Effects of Fiber Type and Size on the Heterogeneity of Oxygen Distribution in Exercising Skeletal Muscle. PLoS ONE 7, e44375. https://doi.org/10.1371/journal.pone.0044375

Lombard, J.H., Frisbee, J.C., Greene, A.S., Hudetz, A.G., Roman, R.J., Tonellato, P.J., 2000. Microvascular flow and tissue PO(2) in skeletal muscle of chronic reduced renal mass hypertensive rats. Am. J. Physiol. Heart Circ. Physiol. 279, H2295-2302. https://doi.org/10.1152/ajpheart.2000.279.5.H2295

Lui, M.A., Mahalingam, S., Patel, P., Connaty, A.D., Ivy, C.M., Cheviron, Z.A., Storz, J.F., McClelland, G.B., Scott, G.R., 2015. High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. 308, R779-791. https://doi.org/10.1152/ajpregu.00362.2014

Lundby, C., 2004. Acclimatization to 4100 m does not change capillary density or mRNA expression of potential angiogenesis regulatory factors in human skeletal muscle. J. Exp. Biol. 207, 3865–3871. https://doi.org/10.1242/jeb.01225

Lundby, C., Calbet, J.A.L., Robach, P., 2009. The response of human skeletal muscle tissue to hypoxia. Cell. Mol. Life Sci. CMLS 66, 3615–3623. https://doi.org/10.1007/s00018-009-0146-8

Lundby, C., Gassmann, M., Pilegaard, H., 2006. Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. Eur. J. Appl. Physiol. 96, 363–369. https://doi.org/10.1007/s00421-005-0085-5

Lundby, C., Millet, G.P., Calbet, J.A., Bärtsch, P., Subudhi, A.W., 2012. Does "altitude training" increase exercise performance in elite athletes? Br. J. Sports Med. 46, 792–795. https://doi.org/10.1136/bjsports-2012-091231

Lynch, J.M., Maillet, M., Vanhoutte, D., Schloemer, A., Sargent, M.A., Blair, N.S., Lynch, K.A., Okada, T., Aronow, B.J., Osinska, H., Prywes, R., Lorenz, J.N., Mori, K., Lawler, J., Robbins, J., Molkentin, J.D., 2012. A thrombospondin-dependent pathway for a protective ER stress response. Cell 149, 1257–1268. https://doi.org/10.1016/j.cell.2012.03.050

MacDOUGALL, J.D., Green, H.J., Sutton, J.R., Coates, G., Cymerman, A., Young, P., Houston, C.S., 1991. Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. Acta Physiol. Scand. 142, 421–427. https://doi.org/10.1111/j.1748-1716.1991.tb09176.x

Magalhães, F. de C., Aguiar, P.F., Tossige-Gomes, R., Magalhães, S.M., Ottone, V. de O., Fernandes, T., Oliveira, E.M., Dias-Peixoto, M.F., Rocha-Vieira, E., Amorim, F.T., 2020. High-intensity interval training followed by postexercise cold-water immersion does not alter angiogenic circulating cells, but increases circulating endothelial cells. Appl. Physiol. Nutr. Metab. 45, 101–111. https://doi.org/10.1139/apnm-2019-0041

Malek, M.H., Olfert, I.M., 2009. Global deletion of thrombospondin-1 increases cardiac and skeletal muscle capillarity and exercise capacity in mice. Exp. Physiol. 94, 749–760. https://doi.org/10.1113/expphysiol.2008.045989

Malek, M.H., Olfert, I.M., Esposito, F., 2010. Detraining losses of skeletal muscle capillarization are associated with vascular endothelial growth factor protein expression in rats. Exp. Physiol. 95, 359–368. https://doi.org/10.1113/expphysiol.2009.050369

Mardakheh, F.K., Paul, A., Kümper, S., Sadok, A., Paterson, H., Mccarthy, A., Yuan, Y., Marshall, C.J., 2015. Global Analysis of mRNA, Translation, and Protein Localization: Local Translation Is a Key Regulator of Cell Protrusions. Dev. Cell 35, 344–357. https://doi.org/10.1016/j.devcel.2015.10.005

Mathieu-Costello, O., 1994. Morphometry of the Size of the Capillary-to-Fiber Interface in Muscles, in: Vaupel, P., Zander, R., Bruley, D.F. (Eds.), Oxygen Transport to Tissue XV, Advances in Experimental Medicine and Biology. Springer US, Boston, MA, pp. 661–668. https://doi.org/10.1007/978-1-4615-2468-7_87

Mathieu-Costello, O., Agey, P.J., 1997. Chronic hypoxia affects capillary density and geometry in pigeon pectoralis muscle. Respir. Physiol. 109, 39–52. https://doi.org/10.1016/S0034-5687(97)84028-5

Mathieu-Costello, O., Agey, P.J., Quintana, E.S., Rousey, K., Wu, L., Bernstein, M.H., 1998. Fiber capillarization and ultrastructure of pigeon pectoralis muscle after cold acclimation. J. Exp. Biol. 201, 3211–3220.

Maurer, A.-M., Zhou, B., Han, Z.C., 2006. Roles of platelet factor 4 in hematopoiesis and angiogenesis. Growth Factors Chur Switz. 24, 242–252. https://doi.org/10.1080/08977190600988225

McDermott, M.M., Ades, P., Guralnik, J.M., Dyer, A., Ferrucci, L., Liu, K., Nelson, M., Lloyd-Jones, D., Van Horn, L., Garside, D., Kibbe, M., Domanchuk, K., Stein, J.H., Liao, Y., Tao, H., Green, D., Pearce, W.H., Schneider, J.R., McPherson, D., Laing, S.T., McCarthy, W.J., Shroff, A., Criqui, M.H., 2009. Treadmill exercise and resistance training in patients with peripheral arterial disease with and without intermittent claudication: a randomized controlled trial. JAMA 301, 165–174. https://doi.org/10.1001/jama.2008.962

Meissner, J.D., Umeda, P.K., Chang, K.-C., Gros, G., Scheibe, R.J., 2007. Activation of the β myosin heavy chain promoter by MEF-2D, MyoD, p300, and the calcineurin/NFATc1 pathway. J. Cell. Physiol. 211, 138–148. https://doi.org/10.1002/jcp.20916

Milkiewicz, M., Brown, M.D., Egginton, S., Hudlicka, O., 2001. Association between shear stress, angiogenesis, and VEGF in skeletal muscles in vivo. Microcirc. N. Y. N 1994 8, 229–241. https://doi.org/10.1038/sj/mn/7800074

Milkiewicz, M., Doyle, J.L., Fudalewski, T., Ispanovic, E., Aghasi, M., Haas, T.L., 2007. HIF-1α and HIF-2α play a central role in stretch-induced but not shear-stress-induced angiogenesis in rat skeletal muscle: HIF-1α and HIF-2α in stretch-induced angiogenesis. J. Physiol. 583, 753–766. https://doi.org/10.1113/jphysiol.2007.136325

Milkiewicz, M., Roudier, E., Doyle, J.L., Trifonova, A., Birot, O., Haas, T.L., 2011. Identification of a Mechanism Underlying Regulation of the Anti-Angiogenic Forkhead Transcription Factor FoxO1 in Cultured Endothelial Cells and Ischemic Muscle. Am. J. Pathol. 178, 935–944. https://doi.org/10.1016/j.ajpath.2010.10.042

Miller, D.L., Dibbens, J.A., Damert, A., Risau, W., Vadas, M.A., Goodall, G.J., 1998. The vascular endothelial growth factor mRNA contains an internal ribosome entry site. FEBS Lett. 434, 417–420. https://doi.org/10.1016/s0014-5793(98)01025-4

Millet, G.P., Debevec, T., Brocherie, F., Girard, O., Pialoux, V., Wüst, R.C.I., Degens, H., Zuo, L., He, F., Chaillou, T., Cheng, A., Kayser, B., Favier, F.B., Begue, G., Murray, A.J., Horscroft, J.A., 2017. Commentaries on Viewpoint: Human skeletal muscle wasting in hypoxia: a matter of hypoxic dose? J. Appl. Physiol. Bethesda Md 1985 122, 409–411. https://doi.org/10.1152/japplphysiol.01084.2016

Millet, G.P., Debevec, T., Brocherie, F., Malatesta, D., Girard, O., 2016. Therapeutic Use of Exercising in Hypoxia: Promises and Limitations. Front. Physiol. 7. https://doi.org/10.3389/fphys.2016.00224

Millet, G.P., Faiss, R., Pialoux, V., 2013. Evidence for differences between hypobaric and normobaric hypoxia is conclusive. Exerc. Sport Sci. Rev. 41, 133. https://doi.org/10.1097/JES.0b013e318271a5e1

Millet, G.P., Faiss, R., Pialoux, V., 2012a. Last word on Point: Counterpoint: Hypobaric hypoxia induces different responses from normobaric hypoxia. J. Appl. Physiol. Bethesda Md 1985 112, 1795. https://doi.org/10.1152/japplphysiol.00338.2012

Millet, G.P., Faiss, R., Pialoux, V., 2012b. Point: Hypobaric hypoxia induces different physiological responses from normobaric hypoxia. J. Appl. Physiol. Bethesda Md 1985 112, 1783–1784. https://doi.org/10.1152/japplphysiol.00067.2012

Millet, G.P., Roels, B., Schmitt, L., Woorons, X., Richalet, J.P., 2010. Combining Hypoxic Methods for Peak Performance: Sports Med. 40, 1–25. https://doi.org/10.2165/11317920-000000000-00000

Mizuno, M., Juel, C., Bro-Rasmussen, T., Mygind, E., Schibye, B., Rasmussen, B., Saltin, B., 1990. Limb skeletal muscle adaptation in athletes after training at altitude. J. Appl. Physiol. 68, 496–502. https://doi.org/10.1152/jappl.1990.68.2.496

Mizuno, M., Savard, G.K., Areskog, N.-H., Lundby, C., Saltin, B., 2008. Skeletal muscle adaptations to prolonged exposure to extreme altitude: a role of physical activity? High Alt. Med. Biol. 9, 311–317. https://doi.org/10.1089/ham.2008.1009

Molé, P.A., Chung, Y., Tran, T.K., Sailasuta, N., Hurd, R., Jue, T., 1999. Myoglobin desaturation with exercise intensity in human gastrocnemius muscle. Am. J. Physiol. 277, R173-180. https://doi.org/10.1152/ajpregu.1999.277.1.R173

Morandi, V., Petrik, J., Lawler, J., 2021. Endothelial Cell Behavior Is Determined by Receptor Clustering Induced by Thrombospondin-1. Front. Cell Dev. Biol. 9, 664696. https://doi.org/10.3389/fcell.2021.664696

Morfoisse, F., Renaud, E., Hantelys, F., Prats, A.-C., Garmy-Susini, B., 2015. Role of hypoxia and vascular endothelial growth factors in lymphangiogenesis. Mol. Cell. Oncol. 2, e1024821. https://doi.org/10.1080/23723556.2015.1024821

Morici, G., Bonanno, A., Licciardi, A., Valli, G., Passino, C., Bonardi, D., Locorotondo, N., Profita, M., Palange, P., Cogo, A., Bonsignore, M.R., 2013. Plasma leptin and vascular endothelial growth factor (VEGF) in normal subjects at high altitude (5050 m). Arch. Physiol. Biochem. 119, 219–224. https://doi.org/10.3109/13813455.2013.814679

Mounier, R., Brugniaux, J.V., 2012a. Counterpoint: Hypobaric hypoxia does not induce different responses from normobaric hypoxia. J. Appl. Physiol. Bethesda Md 1985 112, 1784–1786. https://doi.org/10.1152/japplphysiol.00067.2012a

Mounier, R., Brugniaux, J.V., 2012b. Last word on Counterpoint: Hypobaric hypoxia does not induce different physiological responses from normobaric hypoxia. J. Appl. Physiol. Bethesda Md 1985 112, 1796. https://doi.org/10.1152/japplphysiol.00355.2012

Muñoz-Chápuli, R., Quesada, A.R., Angel Medina, M., 2004. Angiogenesis and signal transduction in endothelial cells. Cell. Mol. Life Sci. CMLS 61, 2224–2243. https://doi.org/10.1007/s00018-004-4070-7

Murray, A.J., Horscroft, J.A., 2016. Mitochondrial function at extreme high altitude: Mitochondrial function at extreme high altitude. J. Physiol. 594, 1137–1149. https://doi.org/10.1113/JP270079

Nagahisa, H., Mukai, K., Ohmura, H., Takahashi, T., Miyata, H., 2016. Effect of High-Intensity Training in Normobaric Hypoxia on Thoroughbred Skeletal Muscle. Oxid. Med. Cell. Longev. 2016, 1–10. https://doi.org/10.1155/2016/1535367

Nascimento, E.B.M., Hangelbroek, R.W.J., Hooiveld, G.J.E.J., Hoeks, J., Van Marken Lichtenbelt, W.D., Hesselink, M.H.C., Schrauwen, P., Kersten, S., 2020. Comparative transcriptome analysis of human skeletal muscle in response to cold acclimation and exercise training in human volunteers. BMC Med. Genomics 13, 124. https://doi.org/10.1186/s12920-020-00784-z

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z., 1999. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 13, 9–22.

Ng, Y.-S., Rohan, R., Sunday, M. e., Demello, D. e., D'Amore, P. a., 2001. Differential expression of VEGF isoforms in mouse during development and in the adult. Dev. Dyn. 220, 112–121. https://doi.org/10.1002/1097-0177(2000)9999:9999<::AID-DVDY1093>3.0.CO;2-D

Nilles, E., Sayward, H., D'Onofrio, G., 2009. Vascular endothelial growth factor and acute mountain sickness. J. Emerg. Trauma Shock 2, 6–9. https://doi.org/10.4103/0974-2700.44675

Ochoa, C.D., Yu, L., Al-Ansari, E., Hales, C.A., Quinn, D.A., 2010. Thrombospondin-1 null mice are resistant to hypoxia-induced pulmonary hypertension. J. Cardiothorac. Surg. 5, 32. https://doi.org/10.1186/1749-8090-5-32

Oelz, O., Howald, H., Di Prampero, P.E., Hoppeler, H., Claassen, H., Jenni, R., Buhlmann, A., Ferretti, G., Bruckner, J.C., Veicsteinas, A., et, al., 1986. Physiological profile of world-class high-altitude climbers. J. Appl. Physiol. 60, 1734–1742. https://doi.org/10.1152/jappl.1986.60.5.1734

Olenich, S.A., Audet, G.N., Roberts, K.A., Olfert, I.M., 2014. Effects of detraining on the temporal expression of positive and negative angioregulatory proteins in skeletal muscle of mice. J. Physiol. 592, 3325–3338. https://doi.org/10.1113/jphysiol.2014.271213

Olfert, I.M., 2016. Physiological Capillary Regression is not Dependent on Reducing VEGF Expression. Microcirc. N. Y. N 1994 23, 146–156. https://doi.org/10.1111/micc.12263

Olfert, I.M., Baum, O., Hellsten, Y., Egginton, S., 2015. Advances and challenges in skeletal muscle angiogenesis. Am. J. Physiol.-Heart Circ. Physiol. 310, H326–H336. https://doi.org/10.1152/ajpheart.00635.2015

Olfert, I.M., Birot, O., 2011. Importance of anti-angiogenic factors in the regulation of skeletal muscle angiogenesis. Microcirc. N. Y. N 1994 18, 316–330. https://doi.org/10.1111/j.1549-8719.2011.00092.x

Olfert, I.M., Breen, E.C., Gavin, T.P., Wagner, P.D., 2006. Temporal thrombospondin-1 mRNA response in skeletal muscle exposed to acute and chronic exercise. Growth Factors 24, 253–259. https://doi.org/10.1080/08977190601000111

Olfert, I.M., Breen, E.C., Mathieu-Costello, O., Wagner, P.D., 2001. Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. J. Appl. Physiol. 91, 1176–1184. https://doi.org/10.1152/jappl.2001.91.3.1176

Olfert, I.M., Howlett, R.A., Tang, K., Dalton, N.D., Gu, Y., Peterson, K.L., Wagner, P.D., Breen, E.C., 2009. Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. J. Physiol. 587, 1755–1767. https://doi.org/10.1113/jphysiol.2008.164384

Olfert, I.M., Howlett, R.A., Wagner, P.D., Breen, E.C., 2010. Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 299, R1059-1067. https://doi.org/10.1152/ajpregu.00347.2010

Olsson, A.-K., Dimberg, A., Kreuger, J., Claesson-Welsh, L., 2006. VEGF receptor signalling ? in control of vascular function. Nat. Rev. Mol. Cell Biol. 7, 359–371. https://doi.org/10.1038/nrm1911

Oltmanns, K.M., Gehring, H., Rudolf, S., Schultes, B., Hackenberg, C., Schweiger, U., Born, J., Fehm, H.L., Peters, A., 2006. Acute hypoxia decreases plasma VEGF concentration in healthy humans. Am. J. Physiol.-Endocrinol. Metab. 290, E434–E439. https://doi.org/10.1152/ajpendo.00508.2004

Opichka, M., Shute, R., Marshall, K., Slivka, D., 2019. Effects of exercise in a cold environment on gene expression for mitochondrial biogenesis and mitophagy. Cryobiology 90, 47–53. https://doi.org/10.1016/j.cryobiol.2019.08.007

Osera, C., Martindale, J.L., Amadio, M., Kim, J., Yang, X., Moad, C.A., Indig, F.E., Govoni, S., Abdelmohsen, K., Gorospe, M., Pascale, A., 2015. Induction of VEGFA mRNA translation by CoCl2 mediated by HuR. RNA Biol. 12, 1121–1130. https://doi.org/10.1080/15476286.2015.1085276

Ozawa, K., Kondo, T., Hori, O., Kitao, Y., Stern, D.M., Eisenmenger, W., Ogawa, S., Ohshima, T., 2001a. Expression of the oxygen-regulated protein ORP150 accelerates wound healing by modulating intracellular VEGF transport. J. Clin. Invest. 108, 41–50. https://doi.org/10.1172/JCI11772

Ozawa, K., Tsukamoto, Y., Hori, O., Kitao, Y., Yanagi, H., Stern, D.M., Ogawa, S., 2001b. Regulation of tumor angiogenesis by oxygen-regulated protein 150, an inducible endoplasmic reticulum chaperone. Cancer Res. 61, 4206–4213.

Park, J.E., Keller, G.A., Ferrara, N., 1993. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol. Biol. Cell 4, 1317–1326. https://doi.org/10.1091/mbc.4.12.1317

Patitucci, M., Lugrin, D., Pagès, G., 2009. Angiogenic/lymphangiogenic factors and adaptation to extreme altitudes during an expedition to Mount Everest. Acta Physiol. 196, 259–265. https://doi.org/10.1111/j.1748-1716.2008.01915.x

Paudyal, A., Slevin, M., Maas, H., Degens, H., 2018. Time course of denervation-induced changes in gastrocnemius muscles of adult and old rats. Exp. Gerontol. 106, 165–172. https://doi.org/10.1016/j.exger.2018.03.008

Phelan, M.W., Forman, L.W., Perrine, S.P., Faller, D.V., 1998. Hypoxia increases thrombospondin-1 transcript and protein in cultured endothelial cells. J. Lab. Clin. Med. 132, 519–529. https://doi.org/10.1016/s0022-2143(98)90131-7

Pilotte, J., Cunningham, B.A., Edelman, G.M., Vanderklish, P.W., 2009. Developmentally regulated expression of the cold-inducible RNA-binding motif protein 3 in euthermic rat brain. Brain Res. 1258, 12–24. https://doi.org/10.1016/j.brainres.2008.12.050

Pilotte, J., Kiosses, W., Chan, S.W., Makarenkova, H.P., Dupont-Versteegden, E., Vanderklish, P.W., 2018. Morphoregulatory functions of the RNA-binding motif protein 3 in cell spreading, polarity and migration. Sci. Rep. 8, 7367. https://doi.org/10.1038/s41598-018-25668-2

Poole, D.C., Kano, Y., Koga, S., Musch, T.I., 2021. August Krogh: Muscle capillary function and oxygen delivery. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 253, 110852. https://doi.org/10.1016/j.cbpa.2020.110852

Poole, D.C., Mathieu-Costello, O., 1996. Relationship between fiber capillarization and mitochondrial volume density in control and trained rat soleus and plantaris muscles. Microcirc. N. Y. N 1994 3, 175–186. https://doi.org/10.3109/10739689609148286

Poole, D.C., Mathieu-Costello, O., 1989. Skeletal muscle capillary geometry: adaptation to chronic hypoxia. Respir. Physiol. 77, 21–29. https://doi.org/10.1016/0034-5687(89)90026-1

Poole, D.C., Pittman, R.N., Musch, T.I., Østergaard, L., 2020. August Krogh's theory of muscle microvascular control and oxygen delivery: a paradigm shift based on new data. J. Physiol. 598, 4473–4507. https://doi.org/10.1113/JP279223

Pramsohler, S., Burtscher, M., Faulhaber, M., Gatterer, H., Rausch, L., Eliasson, A., Netzer, N.C., 2017. Endurance Training in Normobaric Hypoxia Imposes Less Physical Stress for Geriatric Rehabilitation. Front. Physiol. 8, 514. https://doi.org/10.3389/fphys.2017.00514

Prewitt, R.L., Johnson, P.C., 1976. The effect of oxygen on arteriolar red cell velocity and capillary density in the rat cremaster muscle. Microvasc. Res. 12, 59–70. https://doi.org/10.1016/0026-2862(76)90007-8

Prior, S.J., Goldberg, A.P., Ortmeyer, H.K., Chin, E.R., Chen, D., Blumenthal, J.B., Ryan, A.S., 2015. Increased Skeletal Muscle Capillarization Independently Enhances Insulin Sensitivity in Older Adults After Exercise Training and Detraining. Diabetes 64, 3386–3395. https://doi.org/10.2337/db14-1771

Ramos-Campo, D.J., Girard, O., Pérez, A., Rubio-Arias, J.Á., 2019. Additive stress of normobaric hypoxic conditioning to improve body mass loss and cardiometabolic markers in individuals with overweight or obesity: A systematic review and meta-analysis. Physiol. Behav. 207, 28–40. https://doi.org/10.1016/j.physbeh.2019.04.027

Rich, B., Scadeng, M., Yamaguchi, M., Wagner, P.D., Breen, E.C., 2017. Skeletal myofiber vascular endothelial growth factor is required for the exercise training-induced increase in dentate gyrus neuronal precursor cells. J. Physiol. 595, 5931–5943. https://doi.org/10.1113/JP273994

Richalet, J.-P., 2020a. CrossTalk opposing view: Barometric pressure, independent of P O 2 , is not the forgotten parameter in altitude physiology and mountain medicine. J. Physiol. 598, 897–899. https://doi.org/10.1113/JP279160

Richalet, J.-P., 2020b. Rebuttal from Jean-Paul Richalet. J. Physiol. 598, 903. https://doi.org/10.1113/JP279426

Richard, N.A., Sahota, I.S., Widmer, N., Ferguson, S., Sheel, A.W., Koehle, M.S., 2014. Acute mountain sickness, chemosensitivity, and cardiorespiratory responses in humans exposed to hypobaric and normobaric hypoxia. J. Appl. Physiol. Bethesda Md 1985 116, 945–952. https://doi.org/10.1152/japplphysiol.00319.2013

Richardson, R.S., 2000. Intracellular Po2 and bioenergetic measurements in skeletal muscle: the role of exercise paradigm. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278, R1111-1113. https://doi.org/10.1152/ajpregu.2000.278.4.R1111

Richardson, R.S., Duteil, S., Wary, C., Wray, D.W., Hoff, J., Carlier, P.G., 2006. Human skeletal muscle intracellular oxygenation: the impact of ambient oxygen availability. J. Physiol. 571, 415–424. https://doi.org/10.1113/jphysiol.2005.102327

Richardson, R.S., Leigh, J.S., Wagner, P.D., Noyszewski, E.A., 1999a. Cellular PO2 as a determinant of maximal mitochondrial O(2) consumption in trained human skeletal muscle. J. Appl. Physiol. Bethesda Md 1985 87, 325–331. https://doi.org/10.1152/jappl.1999.87.1.325

Richardson, R.S., Noyszewski, E.A., Kendrick, K.F., Leigh, J.S., Wagner, P.D., 1995. Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. J. Clin. Invest. 96, 1916–1926. https://doi.org/10.1172/JCI118237

Richardson, R.S., Wagner, H., Mudaliar, S.R., Henry, R., Noyszewski, E.A., Wagner, P.D., 1999b. Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise. Am. J. Physiol. 277, H2247-2252. https://doi.org/10.1152/ajpheart.1999.277.6.H2247

Richmond, K.N., Burnite, S., Lynch, R.M., 1997. Oxygen sensitivity of mitochondrial metabolic state in isolated skeletal and cardiac myocytes. Am. J. Physiol.-Cell Physiol. 273, C1613–C1622. https://doi.org/10.1152/ajpcell.1997.273.5.C1613

Richmond, K.N., Shonat, R.D., Lynch, R.M., Johnson, P.C., 1999. Critical PO(2) of skeletal muscle in vivo. Am. J. Physiol. 277, H1831-1840. https://doi.org/10.1152/ajpheart.1999.277.5.H1831

Rodriguez-Manzaneque, J.C., Lane, T.F., Ortega, M.A., Hynes, R.O., Lawler, J., Iruela-Arispe, M.L., 2001. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc. Natl. Acad. Sci. U. S. A. 98, 12485–12490. https://doi.org/10.1073/pnas.171460498

Rogers, N.M., Sharifi-Sanjani, M., Yao, M., Ghimire, K., Bienes-Martinez, R., Mutchler, S.M., Knupp, H.E., Baust, J., Novelli, E.M., Ross, M., St Croix, C., Kutten, J.C., Czajka, C.A., Sembrat, J.C., Rojas, M., Labrousse-Arias, D., Bachman, T.N., Vanderpool, R.R., Zuckerbraun, B.S., Champion, H.C., Mora, A.L., Straub, A.C., Bilonick, R.A., Calzada, M.J., Isenberg, J.S., 2017. TSP1-CD47 signaling is upregulated in clinical pulmonary hypertension and contributes to pulmonary arterial vasculopathy and dysfunction. Cardiovasc. Res. 113, 15–29. https://doi.org/10.1093/cvr/cvw218

Roudier, E., Chapados, N., Decary, S., Gineste, C., Le Bel, C., Lavoie, J.-M., Bergeron, R., Birot, O., 2009. Angiomotin p80/p130 ratio: a new indicator of exercise-induced angiogenic activity in skeletal muscles from obese and non-obese rats? J. Physiol. 587, 4105–4119. https://doi.org/10.1113/jphysiol.2009.175554

Roudier, E., Forn, P., Perry, M.E., Birot, O., 2012. Murine double minute-2 expression is required for capillary maintenance and exercise-induced angiogenesis in skeletal muscle. FASEB J. 26, 4530–4539. https://doi.org/10.1096/fj.12-212720

Roudier, E., Gineste, C., Wazna, A., Dehghan, K., Desplanches, D., Birot, O., 2010. Angio-adaptation in unloaded skeletal muscle: new insights into an early and muscle type-specific dynamic process. J. Physiol. 588, 4579–4591. https://doi.org/10.1113/jphysiol.2010.193243

Roudier, E., Milkiewicz, M., Birot, O., Slopack, D., Montelius, A., Gustafsson, T., Paik, J.H., DePinho, R.A., Casale, G.P., Pipinos, I.I., Haas, T.L., 2013. Endothelial FoxO1 is an intrinsic regulator of thrombospondin 1 expression that restrains angiogenesis in ischemic muscle. Angiogenesis 16. https://doi.org/10.1007/s10456-013-9353-x

Santocildes, G., Viscor, G., Pagès, T., Ramos-Romero, S., Torres, J.L., Torrella, J.R., 2021. Physiological Effects of Intermittent Passive Exposure to Hypobaric Hypoxia and Cold in Rats. Front. Physiol. 12, 673095. https://doi.org/10.3389/fphys.2021.673095

Schnyder, S., Handschin, C., 2015. Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise. Bone 80, 115–125. https://doi.org/10.1016/j.bone.2015.02.008

Schobersberger, W., Hobisch-Hagen, P., Fries, D., Wiedermann, F., Rieder-Scharinger, J., Villiger, B., Frey, W., Herold, M., Fuchs, D., Jelkmann, W., 2000. Increase in Immune Activation, Vascular Endothelial Growth Factor and Erythropoietin after an Ultramarathon Run at Moderate Altitude. Immunobiology 201, 611–620. https://doi.org/10.1016/S0171-2985(00)80078-9

Schommer, K., Wiesegart, N., Dehnert, C., Mairbäurl, H., Bärtsch, P., 2011. No correlation between plasma levels of vascular endothelial growth factor or its soluble receptor and acute mountain sickness. High Alt. Med. Biol. 12, 323–327. https://doi.org/10.1089/ham.2011.1020

Scott, G.R., Elogio, T.S., Lui, M.A., Storz, J.F., Cheviron, Z.A., 2015. Adaptive Modifications of Muscle Phenotype in High-Altitude Deer Mice Are Associated with Evolved Changes in Gene Regulation. Mol. Biol. Evol. 32, 1962–1976. https://doi.org/10.1093/molbev/msv076

Semenza, G.L., 2014. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu. Rev. Pathol. 9, 47–71. https://doi.org/10.1146/annurev-pathol-012513-104720

Semenza, G.L., 2001. Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance. J. Clin. Invest. 108, 39–40. https://doi.org/10.1172/JCI13374

Semenza, G.L., Wang, G.L., 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. 12, 5447–5454. https://doi.org/10.1128/mcb.12.12.5447

Shute, R., Marshall, K., Opichka, M., Schnitzler, H., Ruby, B., Slivka, D., 2020. Effects of 7°C environmental temperature acclimation during a 3-week training period. J. Appl. Physiol. 128, 768–777. https://doi.org/10.1152/japplphysiol.00500.2019

Sillau, A.H., Aquin, L., Lechner, A.J., Bui, M.V., Banchero, N., 1980. Increased capillary supply in skeletal muscle of guinea pigs acclimated to cold. Respir. Physiol. 42, 233–245. https://doi.org/10.1016/0034-5687(80)90117-6

Sillau, A.H., Banchero, N., 1977. Effects of hypoxia on capillary density and fiber composition in rat skeletal muscle. Pflugers Arch. 370, 227–232. https://doi.org/10.1007/BF00585531

Slopack, D., Roudier, E., Liu, S.T.K., Nwadozi, E., Birot, O., Haas, T.L., 2014. Forkhead BoxO transcription factors restrain exercise-induced angiogenesis. J. Physiol. 592, 4069–4082. https://doi.org/10.1113/jphysiol.2014.275867

Snijders, T., Nederveen, J.P., Verdijk, L.B., Houben, A.J.H.M., Goossens, G.H., Parise, G., van Loon, L.J.C., 2017. Muscle fiber capillarization as determining factor on indices of insulin sensitivity in humans. Physiol. Rep. 5, e13278. https://doi.org/10.14814/phy2.13278

Snyder, G.K., Farrelly, C., Coelho, J.R., 1992. Adaptations in skeletal muscle capillarity following changes in oxygen supply and changes in oxygen demands. Eur. J. Appl. Physiol. 65, 158–163. https://doi.org/10.1007/BF00705074

Stein, I., Itin, A., Einat, P., Skaliter, R., Grossman, Z., Keshet, E., 1998. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. Mol. Cell. Biol. 18, 3112–3119. https://doi.org/10.1128/MCB.18.6.3112

Stephens, J.M., Halson, S., Miller, J., Slater, G.J., Askew, C.D., 2017. Cold-Water Immersion for Athletic Recovery: One Size Does Not Fit All. Int. J. Sports Physiol. Perform. 12, 2–9. https://doi.org/10.1123/ijspp.2016-0095

Storey, J.M., Storey, K.B., 2019. In defense of proteins: Chaperones respond to freezing, anoxia, or dehydration stress in tissues of freeze tolerant wood frogs. J. Exp. Zool. Part Ecol. Integr. Physiol. 331, 392–402. https://doi.org/10.1002/jez.2306

Stroka, D.M., Burkhardt, T., Desbaillets, I., Wenger, R.H., Neil, D.A.H., Bauer, C., Gassmann, M., Candinas, D., 2001. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J. 15, 2445–2453. https://doi.org/10.1096/fj.01-0125com

Sugasawa, T., Mukai, N., Tamura, K., Tamba, T., Mori, S., Miyashiro, Y., Yamaguchi, M., Nissato, S., Ra, Sg., Yoshida, Y., Hoshino, M., Ohmori, H., Kawakami, Y., Takekoshi, K., 2016. Effects of Cold Stimulation on Mitochondrial Activity and VEGF Expression in vitro. Int. J. Sports Med. 37, 766–778. https://doi.org/10.1055/s-0042-102659

Suzuki, J., Gao, M., Ohinata, H., Kuroshima, A., Koyama, T., 1997a. Chronic cold exposure stimulates microvascular remodeling preferentially in oxidative muscles in rats. Jpn. J. Physiol. 47, 513–520. https://doi.org/10.2170/jjphysiol.47.513

Suzuki, J., Gao, M., Yahata, T., Kuroshima, A., Koyama, T., 1997b. Capillary geometry in the soleus muscle of rats cold-acclimatized for 68 generations. Acta Physiol. Scand. 160, 243–250. https://doi.org/10.1046/j.1365-201X.1997.00136.x

Swenson, E.R., Bärtsch, P., 2013. High Altitude: Human Adaptation to Hypoxia. Springer Science & Business Media.

Takahashi, T., Shibuya, M., 1997. The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. Oncogene 14, 2079–2089. https://doi.org/10.1038/sj.onc.1201047

Takahashi, T., Yamaguchi, S., Chida, K., Shibuya, M., 2001. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J. 20, 2768–2778. https://doi.org/10.1093/emboj/20.11.2768

Tang, K., Breen, E.C., Gerber, H.-P., Ferrara, N.M.A., Wagner, P.D., 2004. Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. Physiol. Genomics 18, 63–69. https://doi.org/10.1152/physiolgenomics.00023.2004

Tang, K., Breen, E.C., Wagner, P.D., 2002. Hu protein R-mediated posttranscriptional regulation of VEGF expression in rat gastrocnemius muscle. Am. J. Physiol.-Heart Circ. Physiol. 283, H1497–H1504. https://doi.org/10.1152/ajpheart.00813.2001

Terrados, N., Melichna, J., Sylvén, C., Jansson, E., Kaijser, L., 1988. Effects of training at simulated altitude on performance and muscle metabolic capacity in competitive road cyclists. Eur. J. Appl. Physiol. 57, 203–209. https://doi.org/10.1007/BF00640664

The Physiological Society, 2016. Physiological and psychological responses and adaptation to cold environments, Mike Tipton, The Biomedical Basis of Elite Performance, Session: Environmental impact on function, adaptation & performance during exercise. East Midlands Conference Centre, Nottingham, UK, 6-8 March 2016.

Tissot van Patot, M.C., Leadbetter, G., Keyes, L.E., Bendrick-Peart, J., Beckey, V.E., Christians, U., Hackett, P., 2005. Greater free plasma VEGF and lower soluble VEGF receptor-1 in acute mountain sickness. J. Appl. Physiol. Bethesda Md 1985 98, 1626–1629. https://doi.org/10.1152/japplphysiol.00589.2004

Uchida, C., Nwadozi, E., Hasanee, A., Olenich, S., Olfert, I.M., Haas, T.L., 2015. Muscle-derived vascular endothelial growth factor regulates microvascular remodelling in response to increased shear stress in mice. Acta Physiol. Oxf. Engl. 214, 349–360. https://doi.org/10.1111/apha.12463

van Ekeren, G.J., Sengers, R.C., Stadhouders, A.M., 1992. Changes in volume densities and distribution of mitochondria in rat skeletal muscle after chronic hypoxia. Int. J. Exp. Pathol. 73, 51–60.

Verges, S., Chacaroun, S., Godin-Ribuot, D., Baillieul, S., 2015. Hypoxic Conditioning as a New Therapeutic Modality. Front. Pediatr. 3. https://doi.org/10.3389/fped.2015.00058

Versey, N.G., Halson, S.L., Dawson, B.T., 2013. Water immersion recovery for athletes: effect on exercise performance and practical recommendations. Sports Med. Auckl. NZ 43, 1101–1130. https://doi.org/10.1007/s40279-013-0063-8

Vogt, M., Puntschart, A., Geiser, J., Zuleger, C., Billeter, R., Hoppeler, H., 2001. Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. J. Appl. Physiol. 91, 173–182. https://doi.org/10.1152/jappl.2001.91.1.173

Volpert, O.V., Tolsma, S.S., Pellerin, S., Feige, J.J., Chen, H., Mosher, D.F., Bouck, N., 1995. Inhibition of angiogenesis by thrombospondin-2. Biochem. Biophys. Res. Commun. 217, 326–332. https://doi.org/10.1006/bbrc.1995.2780

Wagatsuma, A., Tamaki, H., Ogita, F., 2006. Sequential Expression of Vascular Endothelial Growth Factor, Flt-1, and KDR/Flk-1 in Regenerating Mouse Skeletal Muscle 55, 8.

Wang, S., Herndon, M.E., Ranganathan, S., Godyna, S., Lawler, J., Argraves, W.S., Liau, G., 2004. Internalization but not binding of thrombospondin-1 to low density lipoprotein receptor-related protein-1 requires heparan sulfate proteoglycans. J. Cell. Biochem. 91, 766–776. https://doi.org/10.1002/jcb.10781

Wang, X., Bove, A.M., Simone, G., Ma, B., 2020. Molecular Bases of VEGFR-2-Mediated Physiological Function and Pathological Role. Front. Cell Dev. Biol. 8, 599281. https://doi.org/10.3389/fcell.2020.599281

Wang, Z., Huang, H., 2013. Platelet factor-4 (CXCL4/PF-4): an angiostatic chemokine for cancer therapy. Cancer Lett. 331, 147–153. https://doi.org/10.1016/j.canlet.2013.01.006

Wellmann, S., Taube, T., Paal, K., Graf V Einsiedel, H., Geilen, W., Seifert, G., Eckert, C., Henze, G., Seeger, K., 2001. Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by LightCycler technology. Clin. Chem. 47, 654–660.

West, J.B., 2012. High-Altitude Medicine. Am. J. Respir. Crit. Care Med. 186, 1229–1237. https://doi.org/10.1164/rccm.201207-1323CI

West, J.B., 2006. Human Limits for Hypoxia: The Physiological Challenge of Climbing Mt. Everest. Ann. N. Y. Acad. Sci. 899, 15–27. https://doi.org/10.1111/j.1749-6632.2000.tb06173.x

Wickler, S.J., 1981. Capillary supply of skeletal muscles from acclimatized white-footed mice Peromyscus. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 241, R357–R361. https://doi.org/10.1152/ajpregu.1981.241.5.R357

Wiesner, S., Haufe, S., Engeli, S., Mutschler, H., Haas, U., Luft, F.C., Jordan, J., 2010. Influences of normobaric hypoxia training on physical fitness and metabolic risk markers in overweight to obese subjects. Obes. Silver Spring Md 18, 116–120. https://doi.org/10.1038/oby.2009.193

Yadav, V., Matsakas, A., Lorca, S., Narkar, V.A., 2014. PGC1β Activates an Antiangiogenic Program to Repress Neoangiogenesis in Muscle Ischemia. Cell Rep. 8, 783–797. https://doi.org/10.1016/j.celrep.2014.06.040

Yoshioka, M., Tanaka, H., Shono, N., Snyder, E.E., Shindo, M., St-Amand, J., 2003. Serial analysis of gene expression in the skeletal muscle of endurance athletes compared to sedentary

men. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 17, 1812–1819. https://doi.org/10.1096/fj.02-1200com

Zak, R.B., Shute, R.J., Heesch, M.W.S., La Salle, D.T., Bubak, M.P., Dinan, N.E., Laursen, T.L., Slivka, D.R., 2017. Impact of hot and cold exposure on human skeletal muscle gene expression. Appl. Physiol. Nutr. Metab. 42, 319–325. https://doi.org/10.1139/apnm-2016-0415

Zhang, X., Guo, Y.L.P., Lin, M., Chen, G., Zou, Y., Lin, C., Yang, L., Guo, P., Lin, M., 2012. Effect of mild hypothermia on breast cancer cells adhesion and migration. Biosci. Trends 6, 313–324. https://doi.org/10.5582/bst.2012.v6.6.313

Zhang, X., Kazerounian, S., Duquette, M., Perruzzi, C., Nagy, J.A., Dvorak, H.F., Parangi, S., Lawler, J., 2009. Thrombospondin-1 modulates vascular endothelial growth factor activity at the receptor level. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 23, 3368–3376. https://doi.org/10.1096/fj.09-131649

Zieger, M.A., Gupta, M.P., Wang, M., 2011. Proteomic analysis of endothelial cold-adaptation. BMC Genomics 12, 630. https://doi.org/10.1186/1471-2164-12-630

Zoll, J., Ponsot, E., Dufour, S., Doutreleau, S., Ventura-Clapier, R., Vogt, M., Hoppeler, H., Richard, R., Flück, M., 2006. Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts. J. Appl. Physiol. 100, 1258–1266. https://doi.org/10.1152/japplphysiol.00359.2005

Zwetsloot, K.A., Westerkamp, L.M., Holmes, B.F., Gavin, T.P., 2008. AMPK regulates basal skeletal muscle capillarization and VEGF expression, but is not necessary for the angiogenic response to exercise. J. Physiol. 586, 6021–6035. https://doi.org/10.1113/jphysiol.2008.159871

Appendices

Appendix A

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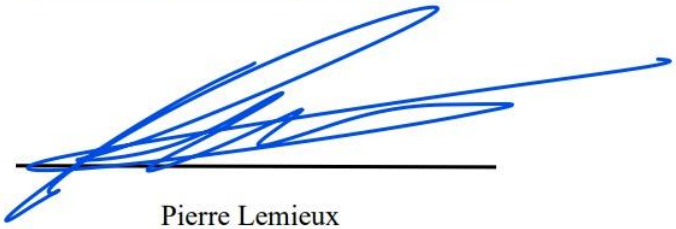
https://doi.org/10.3389/fphys.2021.735557



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Appendix C

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REVIEW
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Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story

*Pierre Lemieux and Olivier Birot**

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Hypoxia, defined as a reduced oxygen availability, can be observed in many tissues in response to various physiological and pathological conditions. As a hallmark of the altitude environment, ambient hypoxia results from a drop in the oxygen pressure in the atmosphere with elevation. A hypoxic stress can also occur at the cellular level when the oxygen supply through the local microcirculation cannot match the cells' metabolic needs. This has been suggested in contracting skeletal myofibers during physical exercise. Regardless of its origin, ambient or exercise-induced, muscle hypoxia triggers complex angio-adaptive responses in the skeletal muscle tissue. These can result in the expression of a plethora of angio-adaptive molecules, ultimately leading to the growth, stabilization, or regression of muscle capillaries. This remarkable plasticity of the capillary network is referred to as angio-adaptation. It can alter the capillary-to-myofiber interface, which represent an important determinant of skeletal muscle function. These angio-adaptive molecules can also be released in the circulation as myokines to act on distant tissues. This review addresses the respective and combined potency of ambient hypoxia and exercise to generate a cellular hypoxic stress in skeletal muscle. The major skeletal muscle angio-adaptive responses to hypoxia so far described in this context will be discussed, including existing controversies in the field. Finally, this review will highlight the molecular complexity of the skeletal muscle angio-adaptive response to hypoxia and identify current gaps of knowledges in this field of exercise and environmental physiology.

Keywords: skeletal muscle, capillary, angiogenesis, VEGF-A, thrombospondin, hypoxia, altitude, exercise

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INTRODUCTION

Our review aims to revisit the complexity of the skeletal muscle angio-adaptive response to hypoxia, particularly when combining exposure to ambient hypoxia and exercise-induced tissue hypoxia. Indeed, in the context of high-altitude expeditions, mountaineers usually engage into prolonged periods of intense physical activity over several weeks or months (West, 2006, 2012). In the context of sport performance at sea level, hypoxia training has become a complex and very specialized area of research (Millet et al., 2010; Lundby et al., 2012; Girard and Chalabi, 2013; Girard and Pluim, 2013; Girard et al., 2013). Finally, the use of exercise training under hypoxia has recently emerged as a new and promising therapeutic avenue to improve some metabolic and cardiovascular conditions

(obesity, type-2 diabetes, hypertension) as well as for the training of elderly subjects (Verges et al., 2015; Millet et al., 2016; **Figure 1**).

Skeletal muscles represent one of our largest tissues, accounting for about 40% of human body weight. Skeletal muscles adapt to environmental, physiological, and pathological conditions with a remarkable plasticity. This can include changes in muscle mass, in the size of myofibers and their metabolic and contractile phenotype, as well as changes in muscle capillarization (Booth and Thomason, 1991; Hudlicka et al., 1992; Hudlicka, 2011).

Since August Krogh's pioneering work about a century ago (Krogh, 1919a,b,c), our understanding of the regulation of muscle blood flow and oxygen delivery to muscle cells has considerably evolved and was recently revisited in great review articles (Angleys and Østergaard, 2020; Poole et al., 2020, 2021; Kissane et al., 2021). The oxygen cascade from skeletal muscle arterioles to capillaries, interstitial tissue, sarcolemma, and mitochondria can be influenced at several levels: The vasomotricity of upstream arterioles and the subsequent regulation of capillary blood flow; the content and velocity of red blood cells; the hemoglobin and myoglobin concentrations; the tortuosity and number of capillaries; and the surface area of myofibers.

The capillary-to-myofiber interface plays a crucial role for muscle function. Indeed, it represents the site of exchange for oxygen, nutrients, metabolic heat and waste between the blood and the myofibers. The density of capillaries within a given area of muscle tissue will greatly contribute to matching the delivery of oxygen and nutrients with the myofibers' metabolic needs, particularly during contractile activity (Hudlicka et al., 1987; Hoppeler and Kayar, 1988; Mathieu-Costello, 1994; Hudlicka, 2011). The capillary network can therefore be considered as a key determinant of skeletal muscle function and several studies have reported strong correlations between the level of muscle capillarization and mitochondria volume density, muscle oxidative capacity, and oxygen consumption (Hoppeler et al., 1987; Hudlicka et al., 1987; Hoppeler and Kayar, 1988; Poole and Mathieu-Costello, 1996; Howlett et al., 2003). For instance, Howlett et al. (2003) reported a strong correlation between skeletal muscle capillary density and muscle oxygen conductance in rats selectively bred for running endurance (Howlett et al., 2003).

SKELETAL MUSCLE CAPILLARIZATION AND THE CONCEPT OF MUSCLE ANGIO-ADAPTATION

The capillary density in a muscle section can vary in response to various environmental, physiological, or pathological conditions. An increase in muscle capillary density is usually observed in human subjects and animal models in response to prolonged endurance training or high-altitude sojourn (Hudlicka et al., 1992; Breen et al., 2008; Hudlicka, 2011). Conversely, skeletal muscle capillary rarefaction has been described in response to physical deconditioning as well as in the context of some pathologies such as chronic obstructive pulmonary disease, chronic heart failure, or diabetes (Roudier et al., 2010; Gouzi et al., 2013; Olfert et al., 2015; Aiken et al., 2019).

Skeletal muscle angio-adaptation refers to the complex and dynamic processes of capillary formation, stabilization, or regression in response to acute and chronic physiological or pathological conditions. These processes are regulated at the molecular level by a plethora of pro- and anti-angiogenic molecules (Hoppeler, 1999; Breen et al., 2008; Egginton, 2009; Olfert and Birot, 2011; Egginton and Birot, 2014; Olfert et al., 2015). At the cellular level, endothelial cells proliferate, migrate, and assemble to form new capillaries in the context of angiogenesis, or conversely, undergo apoptosis during capillary regression. Myofibers can represent an important source of production and release of angio-regulatory molecules such as the well-described pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A).

Importantly, changes in capillary density can also be a direct consequence of alterations in the size of myofibers (hypertrophy or atrophy) without any true capillary loss or formation. Also, no change in capillary density is not necessarily synonymous of an absence of angio-adaptive activity. For example, during myofiber hypertrophy, a formation of new capillaries might occur simply to prevent a decrease in the capillary density that would result from the increase in the myofibers surface area. It is also important to note that some angio-adaptive molecules, such as the pro-angiogenic VEGF-A, are not only required for the growth of capillaries but also to maintain existing ones. Evidence suggests an autocrine expression of VEGF-A being important for endothelial cell survival and vascular homeostasis (Lee et al., 2007; Domigan et al., 2015). Finally, the skeletal muscle could also be seen as an endocrine organ releasing "angio-adaptive myokines" in circulation, potentially affecting distant tissues.

SKELETAL MUSCLE HYPOXIA: IMPACT OF ALTITUDE AND EXERCISE

Hypoxia, defined as a lack of oxygen supply to a given tissue, is a powerful pro-angiogenic stimulus for various cell types and tissues including skeletal muscle (Hoppeler and Vogt, 2001; Semenza, 2001; Breen et al., 2008; Fraisl et al., 2009; Favier et al., 2015). Ambient hypoxia is a hallmark of the altitude environment and results from a drop in the oxygen pressure in the atmosphere with elevation. However, the impact of ambient hypoxia *per se* on skeletal muscle angio-adaptive responses and capillarization still remains a source of scientific debate. A hypoxic stress can also be generated locally at the muscle tissue level in response to intense exercise if oxygen delivery cannot match the metabolic needs of contracting myofibers (**Figure 1**).

Several studies have aimed at determining the oxygen partial pressure (PO_2) cascade in human and animal models using different techniques such as proton magnetic resonance spectroscopy, surface electrodes, microcatheter, and more recently phosphorescence quenching. The intramuscular PO_2 at rest is estimated around 27 mmHg with some variations (10-34 mmHg) among studies (different species, muscles, and techniques), below the microvascular PO_2 but well above the

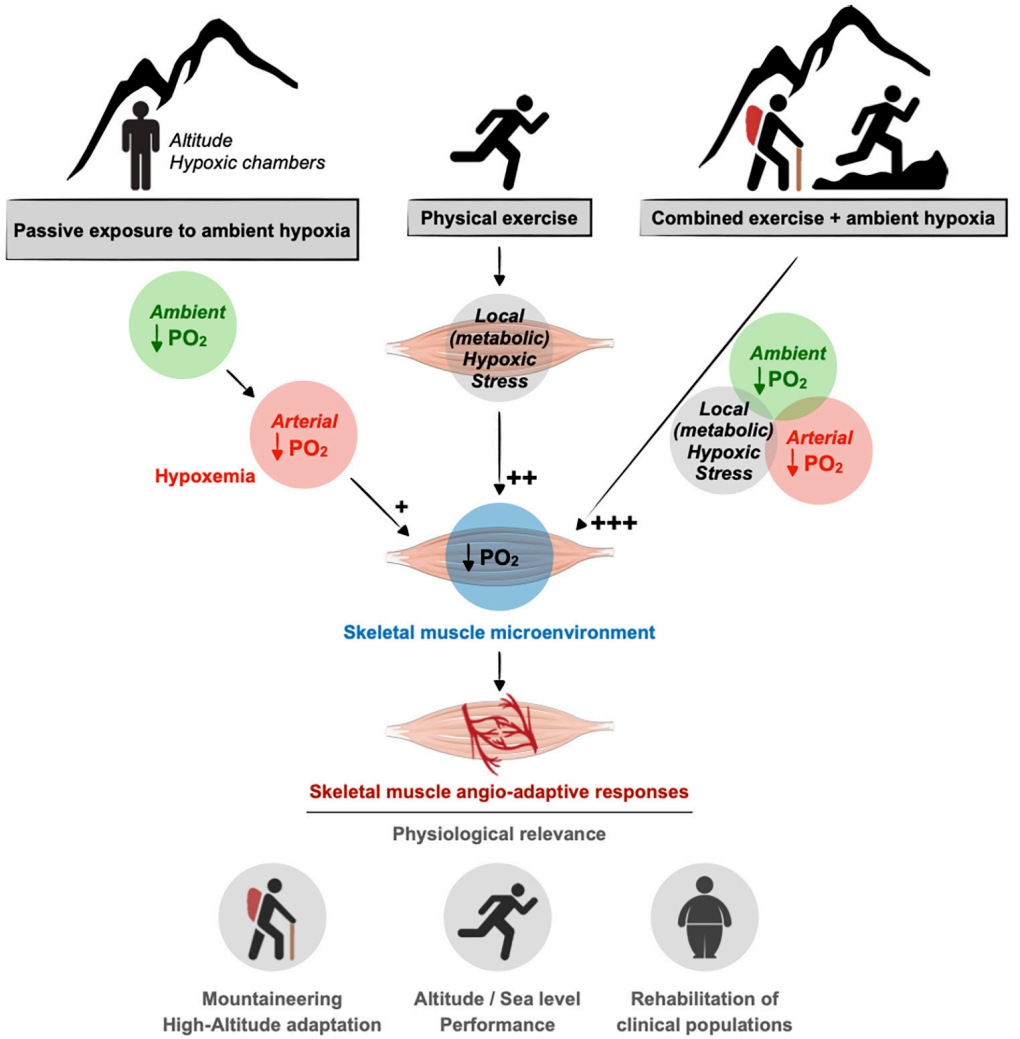

FIGURE 1 | Contribution of ambient hypoxia and exercise to induce a hypoxic stress in skeletal muscle. A drop in the partial pressure of oxygen (PO_2) in the muscle tissue can result from a decrease in ambient PO_2 (e.g., altitude) as well as an unbalanced between blood supply to the muscle cells and their increase metabolic needs during exercise. In response to muscle hypoxia angio-adaptive responses will take place to maintain an optimal oxygen supply to myofibers and to preserve muscle function. This is of particular relevance in the context of mountaineering activity as well as exercise training strategies using ambient hypoxia for athletes or clinical populations. As discussed in the text, the impact of ambient hypoxia on skeletal muscle PO_2 seems rather modest (+) compared to the impact of exercise in generating a local hypoxic stress (++). The combination of ambient hypoxia exposure and exercise might exacerbate this hypoxic stress (+++).

intra-myocyte PO_2 (1-3 mmHg) (Prewitt and Johnson, 1976; Boegehold and Johnson, 1988; Richardson et al., 1995, 2006; Richmond et al., 1997, 1999; Hutter et al., 1999; Molé et al., 1999; Lombard et al., 2000; Richardson, 2000; Kindig et al., 2003; Johnson et al., 2005; Liu et al., 2012; Hirai et al., 2018, 2019; Colburn et al., 2020; Poole et al., 2020, 2021).

A few of these studies have investigated the impact of ambient hypoxia on skeletal muscle PO_2. A modest reduction in PO_2 (from 34 mmHg under normoxia to 23 mmHg under hypoxia) was observed in human quadriceps muscle following an exposure to an inspired O_2 fraction of 10% (F_iO_2 0.10), equivalent to an altitude of about 5,800 m (Richardson et al., 2006). In another study, the interstitial PO_2 in rat cremaster muscles was reduced from 26 to about 10 mmHg following one minute exposure to an inspired O_2 fraction of 7% (F_iO_2 0.07), equivalent to an altitude of about 8,300 m (Johnson et al., 2005). However, the physiological relevance of this conditioning (1 min of exposure at F_iO_2 0.07) can be questioned. These few studies suggest that ambient hypoxia exposure might not alter muscle PO_2 to a large extent. Conversely, the impact of physical exercise on the muscle PO_2 seems much more important. One bout of exercise, even at moderate intensity (50% maximal leg O_2 uptake) was shown to decrease muscle PO_2 to values around 3-5 mmHg (Richardson et al., 1995, 2006; Molé et al., 1999; Angleys and Østergaard, 2020). This exercise-induced hypoxic stress could be further exacerbated (muscle PO_2 down to 2 mmHg) when exercise was performed in a hypoxic environment (Richardson et al., 2006).

At a molecular level, the Hypoxia Inducible Factor-1 (HIF-1) is a transcription factor widely recognized as a hallmark of the hypoxia signaling pathway (Semenza and Wang, 1992; Semenza, 2001). HIF-1 is a heterodimeric complex formed of two subunits alpha and beta. Whereas the expression of the beta subunit remains stable under both normoxic and hypoxic conditions, the alpha subunit (HIF-1α) confers on HIF-1 most of its regulation. Under normoxia, the HIF-1α protein is constantly synthesized in the cytoplasm, and rapidly undergoes proteosomal degradation within a few minutes. This involves HIF-1α hydroxylation on certain proline residues by HIF prolyl hydroxylases (PHD1-3) (Ke and Costa, 2006; Lindholm et al., 2014). If the PO_2 drops enough to generate a hypoxic stress at the cellular level, the function of PHD1-3 is inhibited, HIF-1α is stabilized and can translocate into the nucleus to dimerize with the beta subunit. HIF-1 binds to hypoxia responsive elements (HRE) in the promoter regions of target genes to regulate their transcription. Both the erythropoietin (EPO) and pro-angiogenic VEGF-A genes possess HRE sites in their promoters (Semenza, 2001).

Whether ambient hypoxia exposure results in an increased expression of HIF-1α protein in skeletal muscle remains largely understudied. Stroka et al. (2001) have observed a strong HIF-1α protein expression level in mouse skeletal muscle even under normoxic conditions (Stroka et al., 2001). In the same study, 1 h exposure to extreme normobaric hypoxia (F_iO_2 0.06, equivalent to 9,100 m) did not seem to increase the expression level much further. As noted previously, the physiological relevance of conditionings combining very short exposures (1 min to 1 h) and extreme hypoxia levels (F_iO_2 0.06-0.07) are questionable (Stroka et al., 2001; Johnson et al., 2005).

Intense physical exercise can be at the origin of a local hypoxic stress in the muscle tissue. In an elegant study, Ameln et al. (2005) quantified HIF-1α protein expression in response to one single bout of moderate intensity exercise in human vastus lateralis muscle biopsies (Ameln et al., 2005). HIF-1α protein levels were increased immediately after exercise and remained elevated for up to 6 h post-exercise. An increased nuclear staining for HIF-1α was also observed as well as an increased DNA binding to HRE binding sites. mRNA levels for HIF-1 target genes EPO and VEGF-A were also higher post-exercise. These results suggest that HIF-1 expression and activity were both increased post-exercise in human skeletal muscle.

An increase in HIF-1α protein expression could result from the inhibition of HIF-1α protein degradation but also from an increased expression of HIF-1α mRNA and protein translation. Vogt et al. (2001) have measured HIF-1α mRNA in human vastus lateralis muscle biopsies before and after an endurance training program conducted at low or high intensity either under normoxia or normobaric hypoxia (simulated altitude of 3,850 m) (Vogt et al., 2001). HIF-1α mRNA expression was increased after hypoxia training regardless of the training intensity whereas training under normoxia had no effect. Interestingly, VEGF-A mRNA levels were also measured and found to be increased only in the high-intensity hypoxia training group but not under low exercise training conditions. This could reveal a synergetic effect of combining ambient hypoxia and exercise-induced local hypoxia. Here, biopsies were performed at least 24 h post-exercise to assess the effect of prolonged training on HIF-1α basal levels. In line with this study, Lundby et al. (2006) have evaluated the impact of prolonged exercise training on HIF-1α acute response to one single bout of exercise (Lundby et al., 2006). HIF-1α mRNA levels were increased in vastus lateralis biopsies at 6 h post-exercise only in the untrained group. Whether exercise training could blunt the acute HIF-1α response to one single bout of exercise was then questioned by Lindholm et al. (2014), Lindholm and Rundqvist (2016) who showed that several inhibitors of HIF-1α expression and HIF-1 activity were increased in trained muscles. The analysis of trained muscle biopsies indeed revealed higher expression levels of prolyl hydroxylases (PHD1-3), Factor Inhibiting HIF-1 (FIH) and Sirtuin-6 (SIRT6) (Lindholm et al., 2014). By catalyzing hydroxylation on specific prolyl residues, PHD1-3 target HIF-1α for proteasomal degradation. FIH, a HIF-1α-specific asparagine hydroxylase, can inhibit HIF-1 transactivation. SIRT6, a histone deacetylase, can act as an epigenetic co-repressor of HIF-1. Another explanation to the results from Lundby et al. (2006) could be an increased level of muscle capillarization post-training. Angiogenesis is a well-described tissular adaptation of the skeletal muscle tissue to endurance training. This would result in a better capillary-to-myofiber interface and oxygen delivery to contracting myofibers, and as such, a reduced exercise-induced hypoxic stress in trained muscles.

In addition to inducing a local cellular hypoxic stress, exercise combines other pro-angiogenic stimuli such as increased shear stress on endothelial cells due to enhanced muscle blood flow, mechanical tissue stretch, and oxidative stress (Hudlicka et al., 1992; Milkiewicz et al., 2001; Breen et al., 2008; Egginton, 2009; Hoier et al., 2012; Egginton and Birot, 2014; Hellsten and Hoier, 2014; Haas and Nwadozi, 2015; Olfert et al., 2015). The increase in muscle blood flow results from active vasodilation, and the resultant increase in shear stress represents a well-described pro-angiogenic stimulus for skeletal muscle endothelial cells, mediating the production of angio-adaptive molecules such as nitric oxide, metalloproteinases, and VEGF-A (Milkiewicz et al., 2001, 2011; Egginton, 2009, 2011; Hudlicka and Brown, 2009; Hellsten and Hoier, 2014; Haas and Nwadozi, 2015). If local cellular hypoxia and shear stress are often presented side by side as exercise-induced pro-angiogenic stimuli, passive exposure to ambient hypoxia also stimulates vasodilation and increases blood flow in skeletal muscle (Casey and Joyner, 2011, 2012; Joyner and Casey, 2014; Dinenno, 2016). Therefore, ambient hypoxia could then represent an upstream stimulus of muscle shear stress. The degree of vasodilation observed seems linked to the degree of ambient hypoxia, at least for acute exposures. Interestingly, the combination of ambient hypoxia exposure and exercise seems to synergistically induce greater vasodilation and muscle blood flow than what could be expected by simply adding their respective contributions (Casey and Joyner, 2011, 2012; Joyner and Casey, 2014). Finally, to add more complexity, HIF-1α expression can also be stimulated under normoxic conditions in skeletal muscle in response to increased shear stress or mechanical tissue stretch (Milkiewicz et al., 2007).

ALTITUDE, EXERCISE, AND SKELETAL MUSCLE HYPOXIA: KEY POINTS

- Exercise is a more powerful stimulus than ambient hypoxia to decrease muscle PO_2.
- Combining ambient hypoxia and exercise might further decrease muscle PO_2.
- Hypoxia Inducible Factor-1 is a well-established hallmark of hypoxia signaling.
- Skeletal muscle angio-adaptive activity can locally change the level of muscle capillarization and can also release angio-adaptive myokines in the circulation for distant effects.

INCREASED MUSCLE CAPILLARIZATION IN RESPONSE TO EXERCISE TRAINING AND AMBIENT HYPOXIA

We previously discussed how increasing the capillary-to-myofiber interface could be beneficial for maintaining or improving muscle function when oxygen delivery becomes a challenge. The capillary density (CD), which represents the number of capillaries per surface unit of tissue, is often used to assess the level of muscle capillarization (Andersen, 1975; Hudlicka et al., 1992). The CD can however be influenced both by changes in the number of capillaries and alterations in the size of myofibers. Changes in CD might therefore not always be representative of angiogenesis or capillary regression. Conversely, the capillary-to-fiber ratio (C/F) represents one of the best histological parameters to truly appreciate capillaries formation or rarefaction.

Exercise training is a powerful and well-established pro-angiogenic stimulus for the skeletal muscle tissue (Andersen, 1975; Andersen and Henriksson, 1977; Hudlicka et al., 1992; Egginton, 2009). Human and animal studies have shown that one single bout of exercise leads to the production and release of several angio-adaptive molecules both in the muscle microenvironment, for example to stimulate skeletal muscle endothelial cells (Roudier et al., 2012; Aiken et al., 2016), and in the circulation as myokines (Hudlicka et al., 1992; Vogt et al., 2001; Egginton, 2009; Olfert and Birot, 2011; Hoier et al., 2012; Egginton and Birot, 2014). During prolonged exercise training, the chronic repetition of these exercise-induced angiogenic responses can ultimately lead to the formation of new capillaries. This increase in muscle capillarization will usually be reflected by higher C/F and CD in trained muscles (Andersen, 1975; Andersen and Henriksson, 1977; Hudlicka et al., 1992; Egginton, 2009). As previously mentioned, the exercise stimulus combines several stressors such as local tissue hypoxia, increased shear stress, tissue stretch, and oxidative stress. The exact contribution of hypoxia *per se* during exercise remains difficult to study and still unclear. Conversely to regular exercise, muscle hypokinesia or deconditioning can lead to capillary regression (Fujino et al., 2005; Egginton, 2009; Malek et al., 2010; Roudier et al., 2010; Olfert and Birot, 2011).

The effect of passive exposure to ambient hypoxia on skeletal muscle capillarization is less clear than the impact of exercise training. A well-established consensus of the literature is that prolonged exposure to field or simulated high altitude hypoxia results in improved skeletal muscle capillarization and increased capillary density. This alteration in capillary density seems however mainly due to the atrophy of myofibers rather than a true angiogenic response. Several studies have indeed reported an increase in CD concomitantly to a decrease in the myofiber surface area, with no change in the C/F ratio (Oelz et al., 1986; Green et al., 1989; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Kayser et al., 1996).

This should be taken with a certain caution since there are in fact almost as many original studies reporting an increase in muscle CD as studies showing no change (**Figure 2A**; Banchero et al., 1976; Sillau and Banchero, 1977; Oelz et al., 1986; Poole and Mathieu-Costello, 1989; Hoppeler et al., 1990a,b; Bigard et al., 1991; Green et al., 1992; Kayser et al., 1996; Olfert et al., 2001; Lundby, 2004; Mizuno et al., 2008; Levett et al., 2012). We searched the PubMed database for original research studies that analyzed skeletal muscle CD and C/F in animals or human subjects passively exposed to normobaric or hypobaric hypoxia. Studies combining ambient hypoxia and exercise interventions were included only if they had all the required experimental groups to assess the impact of passive hypoxia exposure independently of the exercise training stimulus. We identified a total of 22 original research studies (**Figure 2**). Interestingly, 55% reported a significant increase or a trend for an increase in CD by at least 10% whereas 45% showed no change (**Figure 2A**).

The lack of change in CD could be attributed to an absence of muscle atrophy, and some authors have in fact observed no reduction in myofiber size at high altitude (Green et al., 1992; Lundby, 2004; Levett et al., 2012; D'Hulst et al., 2016). It was then suggested that both the exposure duration and the altitude level could determine whether myofibers would atrophy or not, leading to the notion of hypoxic dose (D'Hulst and Deldicque, 2017; Millet et al., 2017). Some conditionings were indeed performed at moderate altitude (3,000-4,000 m) over 2-3 weeks only, as opposed to longer exposures (8-10 weeks) at higher altitudes (>5,200 m) (Oelz et al., 1986; Green et al., 1989, 1992; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Lundby, 2004; Mizuno et al., 2008; Levett et al., 2012).

It is difficult to identify the source of discrepancy between these studies regarding myofiber size and CD. In rodent studies for example, the age of the animals can be a confounding factor if they are still growing (Banchero, 1985; Snyder et al., 1992). Calorie intake can also influence body and muscle weights. Prolonged mountaineering expeditions at high-altitude can involve logistical constraints for proper nutrition and gastroenteritis disorders are often described (West, 2012; Swenson and Bärtsch, 2013). Hypophagia has been well observed in rodents exposed to prolonged hypoxia, usually requiring the use of pair-fed control animals (Daneshrad et al., 2001, 2003).

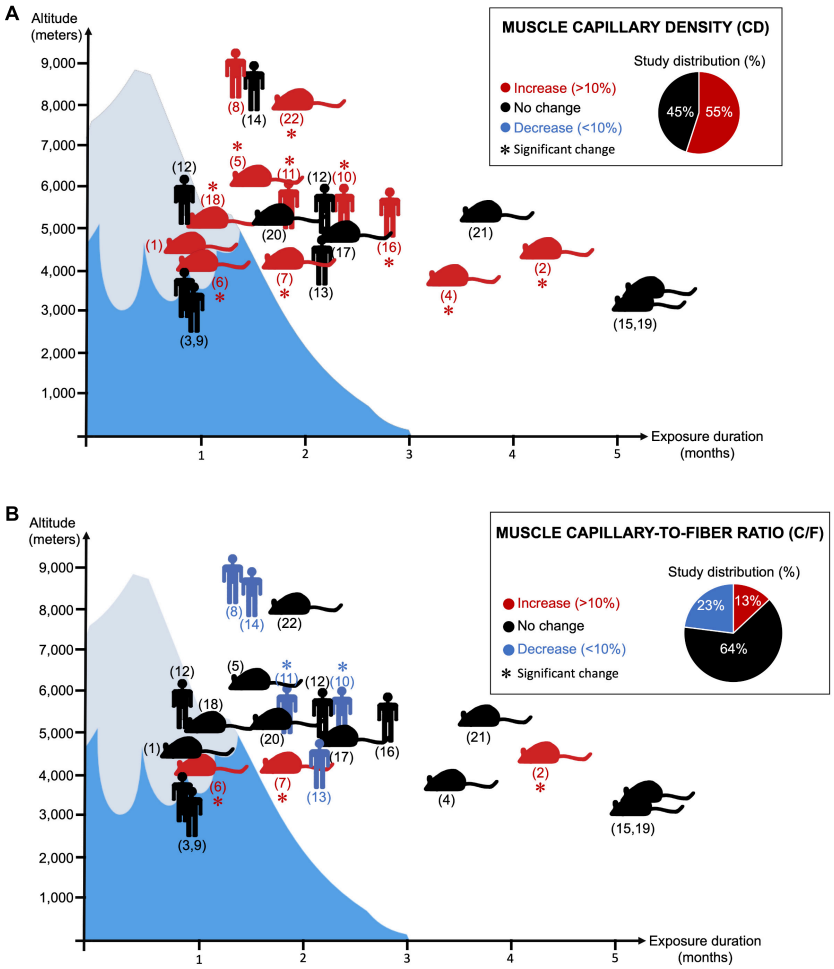

FIGURE 2 | Twenty-two research articles analyzing changes in skeletal muscle capillary density (CD) (Panel **A**) or capillary-to-fiber ratio (C/F) (Panel **B**) in response to prolonged exposure to ambient hypoxia were surveyed. (🧍) indicates human studies. (🐀) indicates the use of animal models (not restricted to rodents). (*) indicates significant finding. Red coloration indicates an increase in magnitude by 10% or greater. Blue coloration indicates a decrease in magnitude by 10% or greater. Black coloration indicates no change. *Y*- and *X*-axis, respectively, reflect the level of altitude (real or simulated) and the duration exposure. Corresponding references: **(1)** Banchero et al. (1976); **(2)** Banchero et al. (1985); **(3)** Basset et al. (2006); **(4)** Bigard et al. (1991); **(5)** Cassin et al. (1971); **(6)** Deveci et al. (2001); **(7)** Deveci et al. (2002); **(8)** Green et al. (1989); **(9)** Green et al. (1992); **(10)** Hoppeler et al. (1990a); **(11)** Hoppeler et al. (1990b); **(12)** Levett et al. (2012); **(13)** Lundby (2004); **(14)** MacDougall et al. (1991); **(15)** Mathieu-Costello and Agey (1997); **(16)** Mizuno et al. (2008); **(17)** Olfert et al. (2001); **(18)** Panisello et al. (2008); **(19)** Poole and Mathieu-Costello (1989); **(20)** Sillau and Banchero (1977); **(21)** Sillau et al. (1980a); **(22)** van Ekeren et al. (1992).

Muscle atrophy could also result from reduced physical activity and muscle deconditioning, particularly during prolonged and passive exposure in hypoxic chambers. For example, when maintaining an isocaloric state and matching physical activity levels, Green et al. (1992) did not observe any atrophy in their study. There is however still a lack of consensus around the contribution of physical activity or the notion of a hypoxic dose on muscle atrophy. This can nicely be illustrated by two studies from Mizuno et al. (2008), Levett et al. (2012). Both compared very active versus less active subjects during similar conditions of prolonged exposure (66-75 days) to high altitude (>5,000 m). Whereas the less active participants remained moderately active at the Everest Base Camp (5,250-5,300 m), active subjects were repeating climbing sessions at higher altitudes. In Levett's study, climbers reached Camp 2 (6,400 m) and some even summited the Mount Everest (8,848 m) (Levett et al., 2012). Mizuno et al. (2008) observed a significant increase in muscle CD (+14%) associated with a reduction in muscle circumferences and myofiber size. Conversely, Levett et al. (2012) did not observe any significant myofiber atrophy or change in CD.

Some authors have also concluded that ambient hypoxia alone might not be sufficient to stimulate skeletal muscle angiogenesis and that a combination of cold and hypoxia stressors might be required (Banchero, 1985; Jackson et al., 1987; Snyder et al., 1992; Hoppeler, 1999). Interestingly, cold *per se* was recently identified as a pro-angiogenic stimulus in rodent skeletal muscle (Sillau et al., 1980b; Egginton, 2002; Deveci and Egginton, 2003).

An increase in muscle CD can also result from the formation of new capillaries via the process of angiogenesis.

The capillary-to-fiber ratio (C/F) is often used to assess capillary formation. Some earlier studies in species adapted to high altitude, including deer mice, gooses, finches or pigeons have reported increased C/F values compared to sea level animals (Mathieu-Costello and Agey, 1997; Hepple et al., 1998; Hepple, 2000; Lui et al., 2015; Scott et al., 2015). Yet, as previously mentioned, a general interpretation is that the increase in CD in response prolonged exposure to hypoxia results from tissue remodeling and myofiber atrophy without any angiogenesis. Methodological artifacts in tissue preparation for histology procedures, such as variation in the sarcomere length, were pointed out (Sillau and Banchero, 1977; Sillau et al., 1980b; Banchero, 1985; Banchero et al., 1985; Hoppeler and Kayar, 1988; Poole and Mathieu-Costello, 1989; Hoppeler et al., 1990a,b; Hudlicka et al., 1992; Mathieu-Costello, 1994). The impact of prolonged hypoxia on muscle C/F was however, revisited by Deveci et al. (2001, 2002) in rats passively exposed to 3 or 6 weeks of hypoxia (F_iO_2 0.12, equivalent to 4,400 m). The effect of hypoxia on C/F could vary accordingly to muscle and fiber types as well as exposure duration. After 3 weeks of conditioning, CD and C/F were increased in the diaphragm and soleus muscles without any significant myofiber atrophy, suggesting a true angiogenic response to ambient hypoxia. No indication of angiogenesis was observed in the tibialis anterior and extensor digitorum longus muscles (Deveci et al., 2001). At 6 weeks, an increase in C/F was observed all muscles (Deveci et al., 2002). Interestingly, the analysis of different areas from each muscle also suggested that angiogenesis might occurs predominantly around larger and glycolytic myofibers (Deveci et al., 2001, 2002).

When re-analyzing our 22 original research studies that measured the C/F ratio in animal and human skeletal muscles in response to prolonged exposure to hypoxia, we found that about 64% mentioned no change and only 13% reported a significant increase or a trend for it (**Figure 2B**). Interestingly, 23% indicated a significant or a trend for a decrease in C/F (Green et al., 1989; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Lundby, 2004). For example, in the study from Hoppeler et al., 1990a,b, the significant decrease in C/F (-10%) is of the same magnitude as the increase in CD (+11%).

Based on the current literature, the understanding of the effect of prolonged exposure to hypoxia on skeletal muscle capillarization is still unclear and difficult to generalize. Responses can vary between species, muscles, and fiber types, and can be different when combining different stressors such as cold or physical activity. The idea of CD increasing because of myofiber atrophy can be seen as an interesting and economic way to improve the capillary-to-myofiber interface without the need of angiogenesis. However, having smaller myofibers might represent a disadvantage when it comes to muscle performance.

Divergent from passive exposure to ambient hypoxia, the utilization of hypoxia in conjunction with exercise has gained popularity as a possible training avenue for improving sea-level exercise performance (Levine, 2002; Levine and Stray-Gundersen, 2006; Millet et al., 2010; Lundby et al., 2012; Chapman, 2013; Girard and Chalabi, 2013; Girard et al., 2013, 2020; Brocherie et al., 2018). As discussed earlier, exercise can generate a local hypoxic stress in the skeletal muscle tissue. It is therefore appealing to consider how the combination of ambient hypoxia and exercise training would affect muscle capillarization.

Several rodent and human studies suggest that skeletal muscle capillarization might improve to a larger extent when exercise training is performed under ambient hypoxia compared to sea level conditions (Terrados et al., 1988; Bigard et al., 1991; van Ekeren et al., 1992; Desplanches et al., 1993, 1996; Olfert et al., 2001; Vogt et al., 2001). To assess the impact of ambient hypoxia on the angiogenic effect of training, some authors trained their animals or subjects at the same relative intensity, for example at a similar percentage of VO_2 max determined under hypoxia and normoxia, thus expecting similar endurance times. Conversely, some authors utilized training protocols with the same absolute intensity, which then can represent a higher stimulus in hypoxic conditions.

Bigard et al. (1991) observed a greater increase in C/F ratio in the plantaris, extensor digitorum longus and soleus muscles of rats housed and trained in a hypobaric chamber at an altitude equivalent to 4,000 m compared to animals trained under normoxic conditions (Bigard et al., 1991). Yet, in the same study C/F values were not different when comparing sedentary normoxic and hypoxic animals, suggesting that hypoxia alone is insufficient to promote muscle angiogenesis. Similarly, Olfert et al. (2001) trained rats for 8 weeks under ambient hypoxia (FiO_2 0.12) or normoxia. The C/F ratio was only increased in muscles from animals trained under hypoxia (Olfert et al., 2001).

Interestingly, Vogt et al. (2001) have also evaluated the effect of exercise intensity during hypoxia training in human subjects. They reported a significant increase in capillarization only in the vastus lateralis muscles of subjects trained at high intensity for 12 weeks under hypoxia (simulated altitude of 3,850 m). Training under normoxia, even at high intensity, did not improve significantly muscle capillarization.

Overall, these laboratory and well-controlled studies indicate a greater angio-adaptive response of the skeletal muscle tissue when exercise training is performed in ambient hypoxia. This reflects into a higher level of muscle capillarization. Yet, it remains unknow whether muscle angio-adaptation occurs faster.

Field studies in humans for prolonged sojourn to altitude focusing on exercise and muscle angiogenic activity have provided mixed results. Mizuno et al. (1990) reported a significant increase in the triceps C/F of competitive cross-country skiers after 2 weeks of training at an altitude of 2,700 m (Mizuno et al., 1990). However, because of the absence of proper control groups (sedentary and trained subjects in normoxia and hypoxia) it cannot really be discerned that these results were simply a response to exercise training. No change in C/F ratio was described in the rectus femoris or biceps brachii of climbers regularly engaged into intense climbing, walking, carrying activities at altitudes above 5,250 m (Mizuno et al., 2008; Levett et al., 2012).

Finally, the use of exercise training in hypoxia for improving certain health outcomes (biomechanical limitations, obesity, hypertension, aging) is also gaining a high interest (Burtscher et al., 2004; Wiesner et al., 2010; Verges et al., 2015; Millet et al., 2016; Kong et al., 2017; Pramsohler et al., 2017; Camacho-Cardenosa et al., 2018, 2019, 2020; Ramos-Campo et al., 2019;

Jung et al., 2021). In regard to skeletal muscle blood flow, capillarization and angio-adaptation, we and others have shown in rodent models and human patients that exercise interventions could represent a powerful therapeutic avenue to prevent, delay or improve alterations in chronic conditions such as peripheral limb ischemia, diabetes, obesity, chronic obstructive pulmonary diseases (Armstrong et al., 1986; Hudlicka et al., 1994; Gardner and Poehlman, 1995; McDermott et al., 2009; Roudier et al., 2009; Gouzi et al., 2013; Amouzou et al., 2016; Aiken et al., 2019). Yet, to the best of our knowledge the impact of hypoxia training as a therapeutic or preventive approach specifically for conditions affecting muscle capillarization and angio-adaptive activity remains to be investigated.

MUSCLE CAPILLARIZATION, EXERCISE TRAINING, AND AMBIENT HYPOXIA: KEY POINTS

- Prolonged exposure to hypoxia can improve skeletal muscle capillary density likely because of myofiber atrophy. Yet the implication of a true angiogenic response cannot be ruled out.
- Combining training and ambient hypoxia can result in a higher muscle angiogenic response than training alone. Yet whether it could also result in an earlier response remains unknown.
- The existing literature obviously shows non-negligible discrepancy imputable sometimes to a lack of proper controls in early studies and to uncontrolled confounding factors such as activity level and training status, cold exposure, restriction in calory intake, animal growth.

SKELETAL MUSCLE CAPILLARIZATION IN HIGH-ALTITUDE NATIVE POPULATIONS

As mentioned previously, a classical idea is that prolonged exposure of lowlanders to high altitude could result in increased skeletal muscle CD often attributed to myofiber atrophy. Could this represent a phenotypic transition towards highlanders' muscles (Gilbert-Kawai et al., 2014)?

Interestingly, CD measured in highlanders' muscles do not seem to differ much from lowlanders. Kayser et al. (1991) have compared the CD from Tibetan Sherpas' muscles with average CD values from sedentary lowlanders or active climbers before and after a high altitude expedition (Kayser et al., 1991). The average CD was significantly higher (+20%) in Sherpas' muscles than in sedentary lowlanders' muscles. However, the training status of the subjects was not taken into consideration. In fact, there was no difference between Sherpas and active climbers before expedition, and a trend for a lower CD (-13%) was even observed in Sherpas' muscles when compared with post-expedition lowlander climbers' muscles (Kayser et al., 1991). It is also important to note that the CD values measured in Sherpas' muscles in this study (Kayser et al., 1991) were compared with CD obtained from different studies (Hoppeler et al., 1990a,b; Oelz et al., 1986). In another study, Kayser et al. (1996) have compared muscles from second-generation Tibetans living at low altitude with Nepalese controls: No difference was observed for muscle CD despite smaller fibers in Tibetans (Kayser et al., 1996).

In an interesting study, Lundby (2004) have compared muscle CD from Aymara subjects, who live permanently around 3,800-4,100 m altitude in Bolivia, with CD values from lowlanders' muscles before and an 8-weeks sojourn at 4,100 m (Lundby, 2004). Aymara muscle CD was 12% (trend) and 15% (significant) lower than lowlanders' CD, respectively, measured before and after the altitude sojourn. This apparent lower CD is intriguing given that high-altitude natives had 25-30% smaller myofibers. The C/F ratio was in fact significantly 40% lower in Aymara's muscles compared to lowlanders.

Altogether, these results suggest that skeletal muscles from high-altitude residents, although presenting smaller fibers, do not have a higher CD than lowlanders' muscles. The observation of a much lower C/F ratio in high-altitude natives reminds the few studies reporting a tendency for a decreased C/F in lowlanders following a prolonged exposure to hypoxia (Green et al., 1989; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Lundby, 2004). It is then tempting to conclude this section by questioning whether a long-term angio-adaptation of the skeletal muscle to hypoxia could in fact result in some capillary regression. Angio-adaptation ensures an optimal match between the muscle capillarization and the metabolic needs of myofibers. If prolonged hypoxia results in an increased CD and smaller myofibers with decreased mitochondria volume density (Horscroft and Murray, 2014; Favier et al., 2015; Murray and Horscroft, 2016; Horscroft et al., 2017), some existing capillaries might at some point become unnecessary.

MOLECULAR ASPECT OF SKELETAL MUSCLE ANGIO-ADAPTIVE RESPONSES TO HYPOXIA

The skeletal muscle capillary network possesses a remarkable plasticity and whether capillaries regress, stabilize or grow in response to a stimulus is largely determined by an intricate balance of pro- and anti-angiogenic molecules (Hoppeler, 1999; Breen et al., 2008; Olfert and Birot, 2011; Egginton and Birot, 2014; Olfert et al., 2015). Among the plethora of angio-adaptive molecules described in the literature, the use of transgenic animal models identified two of them as key regulators of skeletal muscle angio-adaptation: The pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A) (Tang et al., 2004; Olfert et al., 2009, 2010; Baum et al., 2017); and the anti-angiogenic thrombospondin-1 (THBS-1) (Malek and Olfert, 2009; Slopack et al., 2014). Different methodological approaches have been used, some targeting VEGF-A in the whole muscle tissue, some targeting it specifically in myofibers (Tang et al., 2004; Breen et al., 2008; Malek and Olfert, 2009; Olfert et al., 2010; Baum et al., 2017). VEGF-A deletion results in: (1) decreased basal level of muscle capillarization, (2) blunted exercise-induced angiogenic response, (3) severely reduced exercise capacity.

Targeting the anti-angiogenic THBS-1 has somehow opposite consequences: Better vascularized muscles and greater exercise capacity (Breen et al., 2008; Malek and Olfert, 2009; Olfert et al., 2009, 2015). In the context of the present review on skeletal muscle angio-adaptive responses to hypoxia, it is therefore important to recapitulate the response of VEGF-A and THBS-1 to exercise and ambient altitude (**Figure 3**).

Most of studies in exercise and altitude physiology have essentially focused on measuring VEGF-A mRNA and protein expression levels. However, how hypoxia can influence VEGF-A expression and activity is in fact very complex (Semenza, 2001; Breen et al., 2008). The *VEGFA* gene can be considered as a hypoxia-sensitive gene since it possesses several Hypoxia Responsive Elements (HRE) recognized by the transcription factor HIF-1. Under hypoxic conditions, the stabilization of HIF-1α and HIF-1 enhanced transcriptional activity lead to increased VEGF-A mRNA levels (Hoppeler et al., 2003). VEGF-A mRNA can then be stabilized via interaction between its 3' untranslated region and the Human antigen R (HuR protein) (Amadio et al., 2008; Morfoisse et al., 2015; Osera et al., 2015). Interestingly, Tang et al. (2002) have described such interaction between HuR and VEGF-A mRNA in rat ischemic gastrocnemius muscles (Tang et al., 2002). VEGF-A mRNA translation into proteins can also be affected by hypoxia. Indeed, whereas the

FIGURE 3 | Skeletal muscle angio-adaptation is a complex and dynamic process in which much earlier molecular and cellular events lead to eventual changes observed at the tissular level through modifications in the skeletal muscle's capillarization. In response to a given stimulus, whether it be the growth, regression, or stabilization of skeletal muscle capillaries, the process is dictated by an intricate balance of pro- and anti-angiogenic factors. Among the plethora of angio-adaptive factors two have been identified as central in the regulation of skeletal muscle capillarization: the pro-angiogenic VEGF-A; and the anti-angiogenic THBS1. Majority of current literature concerning hypoxic muscle angio-adaptation has focused on the regulation of VEGF-A and as such there are current gaps in knowledge concerning the hypoxic regulation of THBS1. Additionally, there is a lack of consensus regarding the impact of ambient hypoxia eliciting changes at the tissular level concerning CD and C/F.

classical mechanism of mRNA cap-dependent translation can be inhibited under hypoxia, some proteins such as VEGF-A can still be translated by using an alternative translational mechanism where cap-independent translation is initiated via internal ribosome entry sites (IRESs) (Huez et al., 1998; Miller et al., 1998; Stein et al., 1998). The endoplasmic reticulum chaperone Oxygen Regulated Protein-150 (ORP-150) can finally facilitate VEGF-A protein secretion, and an increase in ORP-150 has been reported in rat plantaris muscles following an acute bout of running exercise (Ozawa et al., 2001a,b; Birot et al., 2003). Finally, hypoxia can also stimulate the expression of VEGF-A receptors (Gerber et al., 1997).

A stimulatory effect of exercise on VEGF-A expression has been well described in human and rodent studies. Both mRNA and protein levels are transiently increased after one bout of exercise (Breen et al., 1996, 2008; Gustafsson and Kraus, 2001; Hoppeler and Vogt, 2001; Gustafsson et al., 2002, 2005; Birot et al., 2003; Ameln et al., 2005; Croley et al., 2005; Gavin et al., 2006, 2007; Roudier et al., 2012; Aiken et al., 2016). A study from Breen et al. (1996) has shown that increases in mRNA in rat skeletal muscle were higher and lasted longer when exercise was performed at a higher intensity (Breen et al., 1996). An attenuation of VEGF-A mRNA responsiveness to exercise stimulus has be observed after short-term (few days) training (Gavin and Wagner, 2001; Olfert et al., 2001; Vogt et al., 2001; Kraus et al., 2004).

The effect of acute or chronic exposure to ambient hypoxia itself on VEGF-A expression are less conclusive. Most of the studies that have assessed VEGF-A expression in the muscle tissue have measured mRNA levels only, whereas the protein was essentially quantified in the plasma or serum by ELISA (Asano et al., 1998; Gunga et al., 1999, 2003; Schobersberger et al., 2000; Hanaoka et al., 2003; Oltmanns et al., 2006; Bahtiyar et al., 2007; Dorward et al., 2007; Ding et al., 2012; Morici et al., 2013; Brinkmann et al., 2017; Boos et al., 2018; Kasai et al., 2019; Kasperska and Zembron-Lacny, 2020). Results from the literature remain largely inconsistent, reporting some increase in mRNA or protein (Breen et al., 1996; Gavin et al., 2006), some attenuation (Olfert et al., 2001; Oltmanns et al., 2006; Morici et al., 2013; Boos et al., 2018), or no change (Gunga et al., 2003; Lundby, 2004; Bahtiyar et al., 2007). Additionally, circulating VEGF-A levels measured by ELISA do not necessarily reflect skeletal muscle VEGF-A production.

Finally, some studies have analyzed VEGF-A responsiveness when combining exercise and ambient hypoxia (Breen et al., 1996; Asano et al., 1998; Olfert et al., 2001; Vogt et al., 2001; Gunga et al., 2003; Zoll et al., 2006; Nagahisa et al., 2016; Brocherie et al., 2018; Kasperska and Zembron-Lacny, 2020). Breen et al. (1996) have shown that VEGF-A mRNA expression in rat skeletal muscle was higher and last longer when exercise was performed in ambient hypoxia compared to a normoxic environment (Breen et al., 1996). Brocherie et al. (2018) have compared the effect of different modalities of exercise training combined with hypoxia exposure (Brocherie et al., 2018). VEGF-A mRNA levels were only increased in human skeletal muscle in their model of Live High-Train Low and High (LHTLH) that combined passive exposure to ambient hypoxia and exercise training session at maximal intensity under hypoxia, and no change in VEGF-A was observed in the other two models (Live High-Train Low, LHTL, and Live Low-Train Low, LLTL).This study is in line with previous results reported by Vogt et al. (2001) who analyzed VEGF-A mRNA expression in response to a training program realized either at high or low intensity and either in normoxia or hypoxia (6 weeks at an equivalent of 3,850 m) (Vogt et al., 2001). Whereas training at high intensity in normoxia and training at low intensity in hypoxia only led to a trend for an increase in VEGF-A mRNA (respectively, +13 and +17%), only the combination of training at high intensity in hypoxia resulted in significant increase (+72%). These studies support the idea that combining exercise and ambient hypoxia could exacerbate the angio-adaptive response of the skeletal muscle tissue. If the duration, nature and intensity of the training program are key parameters, all these studies also point out the importance of the duration and intensity of hypoxia exposure. This has recently led to the notion of hypoxic dose (Lundby et al., 2009, 2012; D'Hulst and Deldicque, 2017; Millet et al., 2017; Girard et al., 2020).

Thrombospondin-1 has been identified as a potent anti-angiogenic factor in the skeletal muscle tissue (Malek and Olfert, 2009; Hellsten and Hoier, 2014). Similar to VEGF-A, one bout of exercise stimulates the expression of THBS1mRNA and protein in skeletal muscle (Olfert et al., 2006; Malek and Olfert, 2009; Slopack et al., 2014) whereas short-term training is accompanied by a progressive loss of responsiveness of THBS1 to exercise stimulus (Olfert et al., 2006; Slopack et al., 2014; Hoier et al., 2020; **Figure 3**). This responsiveness, however, appears to be restored with long-term training (Olfert et al., 2006; Hoier et al., 2012; Slopack et al., 2014). The decreased responsiveness of THBS1 during training could in fact contribute to shifting the skeletal muscle angio-adaptive balance towards its pro-angiogenic side, reflected at the tissue level by a pro-angiogenic microenvironment prone to capillary formation. This has led to the hypothesis that exercise-induced angiogenesis might in fact be more controlled by a decrease in anti-angiogenic factors rather than an increase in pro-angiogenic ones (Olfert and Birot, 2011; Hellsten and Hoier, 2014; Olfert et al., 2015; Olfert, 2016). This could apply to capillary regression, as during detraining or muscle hypokinesia, with an increase expression of anti-angiogenic factors shifting the angio-adaptive balance the opposite way (Roudier et al., 2009, 2010; Kishlyansky et al., 2010; Olfert and Birot, 2011; Olenich et al., 2014).

Long-term training does not seem to alter the basal expression level of THBS1 in rodent and human healthy skeletal muscles (Olfert et al., 2006; Hoier et al., 2012; Gliemann et al., 2015) although Yoshioka et al. (2003) have reported higher THBS1 gene expression in skeletal muscles from endurance athletes compared to sedentary subjects (Yoshioka et al., 2003). Interestingly, Gouzi et al. (2013) have shown that muscle THBS1 protein levels could be reduced in response to prolonged training in patients with chronic obstructive pulmonary disease (Gouzi et al., 2013). This reinforces the idea of using exercise training as a therapeutic avenue for clinical conditions with skeletal muscle microvascular alterations. THBS1 expression was indeed found to be increased in rodent skeletal muscles in the context of diabetes, pre-diabetes

and hindlimb ischemia (Kivelä et al., 2006, 2008; Roudier et al., 2013; Dunford et al., 2017; Aiken et al., 2019).

The effect of hypoxia alone on THBS1 expression has provided mixed results essentially from *in vitro* experiments. Phelan et al. (1998) have described an increased expression of THBS1 mRNA and protein in endothelial cells exposed to severe hypoxia (1%O_2) (Phelan et al., 1998). Conversely, Yadav et al. (2014) have observed a decrease in THBS1 expression in differentiated murine C2C12 myotubes in response to hypoxia (Yadav et al., 2014). Finally, THBS1 expression was found to be increased both in rodent and human ischemic skeletal muscles (Roudier et al., 2013).

To our knowledge, there is very limited data regarding THBS1 expression in skeletal muscle when combining exercise stimulus and ambient hypoxia. Olfert et al. (2006) have analyzed THBS1 mRNA expression in skeletal muscles from rats kept sedentary or enrolled into a 8-weeks endurance running program (Olfert et al., 2006). At the end of the training program, animals were performing one bout of intense running exercise. Data suggests that endurance training did not alter the basal expression level of THBS1. However, chronic hypoxia exposure resulted in lower basal expression level (–44%) as well as THBS-1 expression in response to one bout of exercise (–48%).

As a conclusion to this section, it is important to keep in mind that VEGF-A and THBS1 are only two members of the large family of angio-adaptive molecules susceptible to influence the skeletal muscle angio-adaptive responses to exercise and ambient hypoxia. The expression levels of some other angio-adaptive molecules have been measured in skeletal muscle tissue in the context of exposure to ambient hypoxia, such as basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor-β (TGF-β), VEGF receptors, leptin (Breen et al., 1996; Olfert et al., 2001; Patitucci et al., 2009; Morici et al., 2013). However, these measurements remain very anectodical, and more research in this area is needed.

Here, we have also essentially reviewed VEGF-A and THBS1 expression levels, which do not reflect the functionality of these molecules, their interaction between each other's and their receptors.

As illustrated in **Figure 3**, the angio-adaptive response to a given stimulus is a complex and dynamic process that involves molecular, cellular, and tissular responses. There is for example a lack of knowledge regarding cross-talks between muscle cells. For instance, how angio-adaptive signals originating from a contractile myofiber will stimulate neighboring endothelial cells to proliferate and migrate to form new capillaries.

Ideally, evaluating the muscle angio-adaptive response to hypoxia should be integrative. For example, an absence of significant change in capillarization does not rule out any angio-adaptive responses at a cellular or molecular level. Finally, the interpretation of results from the literature is very complex with a certain discrepancy between studies. Such divergence can obviously have several origins: Animal versus human studies; healthy versus pathological conditions; training status; exercise protocols; hypoxia level and duration; confounding environmental stressors (cold, air pollution); time of sample collection; methodology (northern blotting, qPCR, western blotting, ELISA, histochemistry).

THE SKELETAL MUSCLE AS AN ENDOCRINE ORGAN AND THE ROLE OF ANGIO-ADAPTIVE MYOKINES

As previously mentioned, many studies studying angiogenic responses to hypoxia and exercise have quantified circulating VEGF-A protein levels by ELISA. Several studies have aimed to link changes in circulating VEGF-A levels with the susceptibility to develop acute or chronic mountain sickness (Tissot van Patot et al., 2005; Dorward et al., 2007; Nilles et al., 2009; Schommer et al., 2011; Espinoza et al., 2014). Regarding THBS1, this anti-angiogenic molecule seems to be involved in the pathophysiology of hypoxia-induced pulmonary hypertension and right ventricular hypertrophy (Ochoa et al., 2010; Bauer et al., 2012; Rogers et al., 2017). Kaiser et al. (2016) have reported elevated serum THBS1 levels and strong correlations of serum THBS1 to mean pulmonary artery pressure and pulmonary vascular resistance in patients suffering from pulmonary hypertension (Kaiser et al., 2016).

Interestingly, many circulating angio-adaptive molecules, either pro- or anti-angiogenic, could therefore represent valuable biomarkers to evaluate for example the response of athletes to a specific training program in hypoxia or the impact of a therapeutic exercise intervention in a group of patients. More research in identifying the patho-physiological relevance of circulating angio-adaptive biomarkers would be exciting and essential.

The quantification of circulating angio-adaptive molecules also points out the role of the skeletal muscle as an endocrine organ secreting myokines to act on distant organs such as bones, brain, fat, and liver (Fabel et al., 2003; Schnyder and Handschin, 2015; Delezie and Handschin, 2018; Kim et al., 2019; Gomarasca et al., 2020). Fabel et al. (2003) have demonstrated that peripherally produced VEGF-A seems necessary for running-induced improvements in hippocampal neurogenesis (Fabel et al., 2003). Rich et al. (2017) confirmed that VEGF-A produced by skeletal myofibers plays an important role in hippocampal neurogenesis (Rich et al., 2017). Interestingly, it was also suggested that VEGF-A meditated neurogenesis could provide a neuroprotective effect and could be essential for attenuating decrements to cognitive function experienced with ambient hypoxia during high altitude exposure (Koester-Hegmann et al., 2019).

MOLECULAR ASPECT OF SKELETAL MUSCLE ANGIO-ADAPTIVE RESPONSES TO HYPOXIA: KEY POINTS

- Skeletal muscle angio-adaptation is a complex and dynamic process combining molecular and cellular responses that will ultimately alter muscle capillarization.

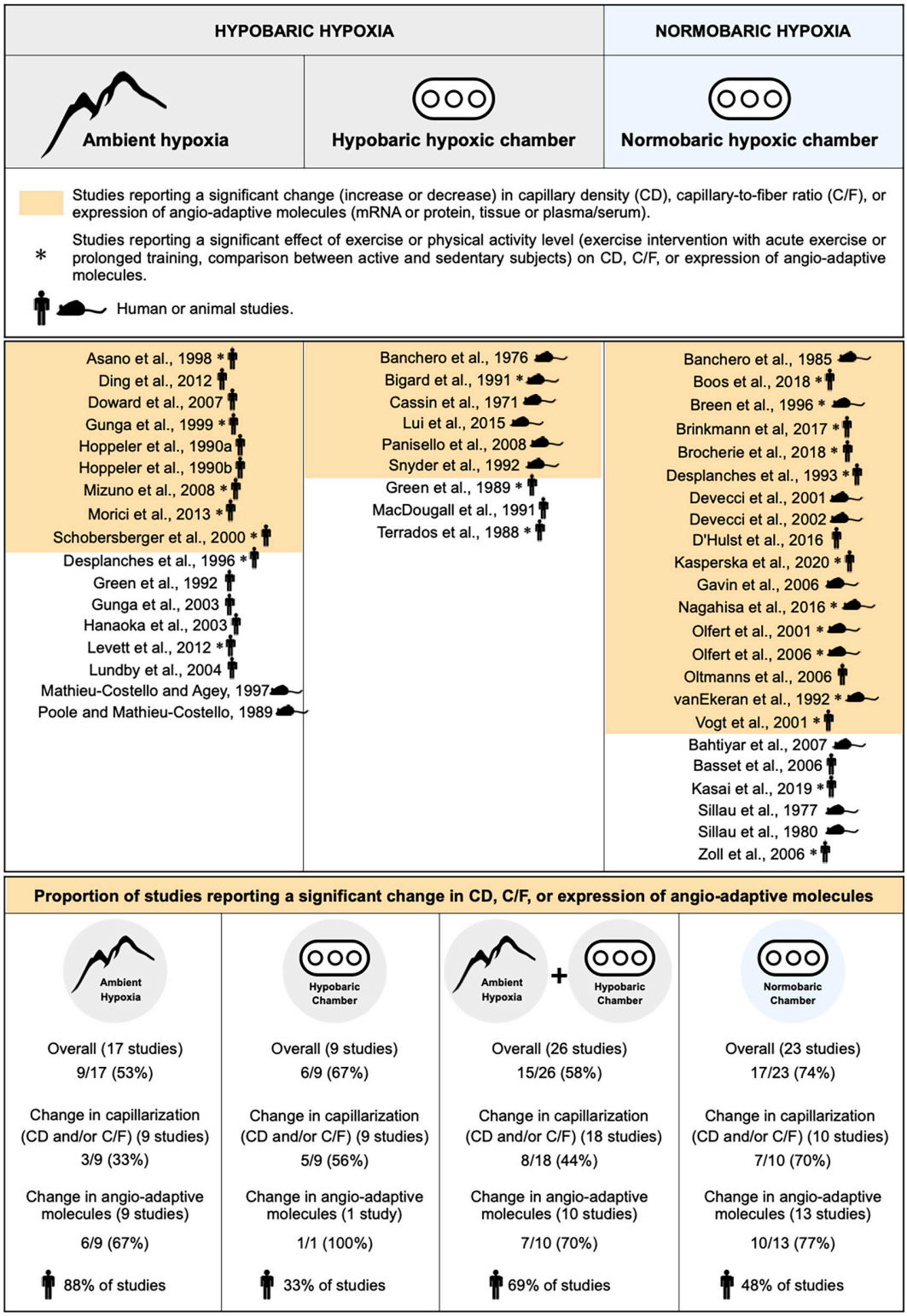

FIGURE 4 | Characterization of the impact of hypobaric and normobaric hypoxia on skeletal muscle angio-adaptive responses. Original research studies cited in the review and analyzing capillary density (CD), capillary-to-fiber ratio (C/F), and expression levels of angio-adaptive molecules in skeletal muscle tissue in response to ambient hypoxia exposure were characterized based on the nature of their hypoxic environment: Hypobaric or normobaric. Studies reporting significant changes in capillarization (CD or C/F), in molecular expression, or in both ("Overall") are highlighted in color. Studies involving a physical activity component are identified by an asterisk. The mouse and human silhouette symbols distinguish studies conducted in animal models or human subjects.

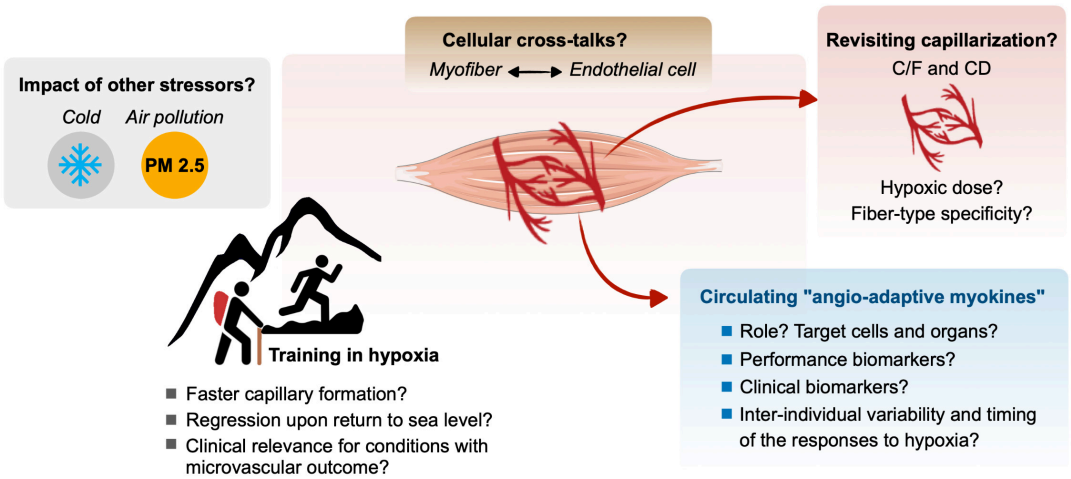

FIGURE 5 | Gap of knowledge and future research directions for a better understanding of the molecular, cellular and tissular angio-adaptive responses of the skeletal muscle tissue to hypoxia, particularly in the context of ambient hypoxia and exercise-induced local hypoxia. Refer to the different text sections for details.

- Several angio-adaptive molecules have been described in the skeletal muscle tissue in the context of exercise-induced angio-adaptation. Yet, their characterization in the context of exposure to ambient hypoxia remains largely unknown.
- There is a current lack of consensus in the literature largely due to confounding experimental variables regarding the expression of muscle VEGF-A and circulating VEGF-A in response to ambient hypoxia alone and to training in hypoxia.
- Anti-angiogenic molecules, such as THBS1, could in fact impact skeletal muscle capillarization to a greater extent than VEGF-A. Their contribution in skeletal muscle angio-adaptive responses to exercise and hypoxia is however, understudied.
- The patho-physiological relevance of circulating angio-adaptive molecules as biomarkers remains poorly documented.
- The role of the skeletal muscle tissue as an endocrine organ secreting angio-adaptive myokines to act on distant organs in the context of exercise and hypoxia is an exciting research avenue.

SKELETAL MUSCLE ANGIO-ADAPTIVE RESPONSES TO HYPOXIA: DO NORMOBARIC AND HYPOBARIC CONDITIONS DIFFER?

The research studies discussed in our review were conducted in three types of environments: Field experiments with real altitude exposure versus hypoxic chambers simulating altitude. Chambers where the barometric pressure and partial oxygen pressure are decreased represent a hypobaric environment, closer to the physics of real altitude, whereas normobaric chambers generate a hypoxic environment by decreasing the fraction of oxygen in the inspired air. Whether normobaric and hypobaric hypoxia are equivalent and then interchangeable has been an intense source of scientific debate, particularly with regards to their impact on exercise performance and various physiological parameters. Systematic reviews and "points-counterpoints" discussions have however, not enable researchers to reach any consensus (Girard et al., 2012; Millet et al., 2012a,b, 2013; Mounier and Brugniaux, 2012a,b; Faiss et al., 2013; Debevec and Millet, 2014; Richard et al., 2014; Coppel et al., 2015; DiPasquale et al., 2016; Richalet, 2020a,b). Discrepancy between studies can be attributable to many cofounding factors: Different degrees of hypoxia, themselves determined either by barometric pressure, inspired PO_2, oxygen fraction; seasonal and geographical differences in barometric pressure; air temperature and humidity; additional environmental stressors such as cold exposure; duration of exposure; presence or not of exercise interventions, and if so different exercise protocols; characteristics of subjects; animal versus human studies.

To the best of our knowledge, whether normobaric and hypobaric hypoxia could differently affect the angio-adaptive responses of the skeletal muscle has never been investigated. We have therefore revisited the original research studies cited in our review that analyzed CD, C/F, or the expression levels of angio-adaptive molecules (mRNA or protein levels, tissue or circulating levels) in response to ambient hypoxia exposure. This represents 49 original studies (**Figure 4**). We separated them accordingly to the hypoxic environment used: Field studies (hypobaric), hypobaric chambers, combined field and hypobaric chambers (both hypobaric environments), and normobaric chambers. Studies involving physical activity (comparing active versus less active subjects, including one bout of exercise or prolonged training) were considered only if they possessed all required

control groups enabling the evaluation of the impact of ambient hypoxia *per se*. We also distinguished studies conducted in animal models or human subjects. Finally, we determined in each category the percentage of studies reporting significant changes (increase or decrease) in capillarization (CD and/or C/F) and expression of angio-adaptive molecules.

Unfortunately, the information presented in **Figure 4** does not really help in answering the question on whether normobaric and hypobaric hypoxia could differently affect the angio-adaptive responses of the skeletal muscle. Indeed, when considering only the studies reporting significant changes in capillarization, a distinction could be made between those conducted in normobaric chambers (70% presenting significant changes) versus studies run in a hypobaric environment (33% for field studies, 56% for hypobaric chambers, and 44% for combined hypobaric environments). However, no such distinction could be made when considering only the studies reporting significant changes in angio-adaptive molecules (67% for field studies, 70% for combined hypobaric environments, and 77% for studies conducted in normobaric chambers). Finally, when looking at "overall" changes (capillarization and angio-adaptive molecules), it seems that a larger proportion of studies conducted in hypoxic chambers, whether normobaric or hypobaric, report significant changes (respectively, 74% and 67%) compared to field studies (53%). As mentioned earlier, a possible explanation could be that field studies often present cofounding factors. Another interesting observation from **Figure 4** is that these conditionings in hypoxic chambers were mainly performed in animal models whereas field studies were essentially involving human subjects (respectively, 33% and 48% of human studies involving hypobaric and normobaric chambers versus 88% for field studies). Similar durations of exposure to hypoxia will obviously not represent the same proportion of a lifespan between rodents and humans.

Based on this analysis, we do not believe that any strong consensus can be established regarding the impact of hypobaric versus normobaric hypoxia on skeletal muscle angio-adaptive responses.

CONCLUSION

Hypoxia, defined as a reduction of oxygen availability can occur in the skeletal muscle tissue of an individual exposed to ambient hypoxia as well as during physical exercise if the oxygen supply to contracting myofibers cannot match their increased metabolic needs. The superimposition of these two stressors, ambient hypoxia exposure and exercise-induced local hypoxia, can lead to an exacerbation of the hypoxic stress experienced by the skeletal muscle. The capillary-to-myofiber interface serves as the site for the exchange of oxygen, nutrients, metabolic heat, and waste between the blood and myofibers. As such, the capillary microvasculature is tightly related to the functional capacity of the skeletal muscle. The capillary microvasculature is a highly adaptive tissue with remarkably plasticity that can grow or regress to various physiological, pathological, and environmental stressors, a process named angio-adaptation. Skeletal muscle angio-adaptation involves complex and dynamic molecular and cellular responses. Given the relevance of skeletal muscle angio-adaptation in response to hypoxia to mountaineers, athletes, and clinical populations, this review aimed to delineate the existing literature and identify current gaps in the knowledge of this field of environmental and exercise physiology (**Figure 5**).

AUTHOR CONTRIBUTIONS

Both authors PL and OB have equally contributed to the design and writing of the manuscript and its figures.

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REFERENCES

Aiken, J., Mandel, E. R., Riddell, M. C., and Birot, O. (2019). Hyperglycaemia correlates with skeletal muscle capillary regression and is associated with alterations in the murine double minute-2/forkhead box O1/thrombospondin-1 pathway in type 1 diabetic BioBreeding rats. *Diab. Vasc. Dis. Res.* 16, 28–37. doi: 10.1177/1479164118805928

Aiken, J., Roudier, E., Ciccone, J., Drouin, G., Stromberg, A., Vojnovic, J., et al. (2016). Phosphorylation of murine double minute-2 on Ser ^{166} is downstream of VEGF-A in exercised skeletal muscle and regulates primary endothelial cell migration and *FoxO* gene expression. *FASEB J.* 30, 1120–1134. doi: 10.1096/fj.15-276964

Amadio, M., Scapagnini, G., Lupo, G., Drago, F., Govoni, S., and Pascale, A. (2008). PKCbetaII/HuR/VEGF: a new molecular cascade in retinal pericytes for the regulation of VEGF gene expression. *Pharmacol. Res.* 57, 60–66. doi: 10.1016/j.phrs.2007.11.006

Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poellinger, L., et al. (2005). Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J.* 19, 1009–1011. doi: 10.1096/fj.04-2304fje

Amouzou, C., Breuker, C., Fabre, O., Bourret, A., Lambert, K., Birot, O., et al. (2016). Skeletal muscle insulin resistance and absence of inflammation characterize insulin-resistant grade I obese women. *PLoS One* 11:e0154119. doi: 10.1371/journal.pone.0154119

Andersen, P. (1975). Capillary density in skeletal muscle of man. *Acta Physiol. Scand.* 95, 203–205. doi: 10.1111/j.1748-1716.1975.tb10043.x

Andersen, P., and Henriksson, J. (1977). Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J. Physiol.* 270, 677–690. doi: 10.1113/jphysiol.1977.sp011975

Angleys, H., and Østergaard, L. (2020). Krogh's capillary recruitment hypothesis, 100 years on: is the opening of previously closed capillaries necessary to ensure muscle oxygenation during exercise? *Am. J. Physiol. Heart Circ. Physiol.* 318, H425–H447. doi: 10.1152/ajpheart.00384.2019

Armstrong, R. B., Ianuzzo, C. D., and Laughlin, M. H. (1986). Blood flow and glycogen use in hypertrophied rat muscles during exercise. *J. Appl. Physiol.* 61, 683–687. doi: 10.1152/jappl.1986.61.2.683

Asano, M., Kaneoka, K., Nomura, T., Asano, K., Sone, H., Tsurumaru, K., et al. (1998). Increase in serum vascular endothelial growth factor levels during altitude training. *Acta Physiol. Scand.* 162, 455–459. doi: 10.1046/j.1365-201X.1998.0318e.x

Bahtiyar, M. O., Buhimschi, C., Ravishankar, V., Copel, J., Norwitz, E., Julien, S., et al. (2007). Contrasting effects of chronic hypoxia and nitric oxide synthase inhibition on circulating angiogenic factors in a rat model of growth restriction. *Am. J. Obstet. Gynecol.* 196, 72.e1–72.e6. doi: 10.1016/j.ajog.2006.07.048

Banchero, N. (1985). Adaptation of muscle capillarity. *Adv. Exp. Med. Biol.* 191, 355–363. doi: 10.1007/978-1-4684-3291-6_36

Banchero, N., Gimenez, M., Rostami, A., and Eby, S. H. (1976). Effects of simulated altitude on O2 transport in dogs. *Respir. Physiol.* 27, 305–321. doi: 10.1016/0034-5687(76)90060-8

Banchero, N., Kayar, S. R., and Lechner, A. J. (1985). Increased capillarity in skeletal muscle of growing guinea pigs acclimated to cold and hypoxia. *Respir. Physiol.* 62, 245–255. doi: 10.1016/0034-5687(85)90118-5

Basset, F. A., Joanisse, D. R., Boivin, F., St-Onge, J., Billaut, F., Doré, J., et al. (2006). Effects of short-term normobaric hypoxia on haematology, muscle phenotypes and physical performance in highly trained athletes. *Exp. Physiol.* 91, 391–402. doi: 10.1113/expphysiol.2005.031682 doi: 10.1113/expphysiol.2005.031682

Bauer, P. M., Bauer, E. M., Rogers, N. M., Yao, M., Feijoo-Cuaresma, M., Pilewski, J. M., et al. (2012). Activated CD47 promotes pulmonary arterial hypertension through targeting caveolin-1. *Cardiovasc. Res.* 93, 682–693. doi: 10.1093/cvr/cvr356

Baum, O., Jentsch, L., Odriozola, A., Tschanz, S. A., and Olfert, I. M. (2017). Ultrastructure of skeletal muscles in mice lacking muscle-specific VEGF expression. *Anat. Rec.* 300, 2239–2249. doi: 10.1002/ar.23644

Bigard, A. X., Brunet, A., Guezennec, C. Y., and Monod, H. (1991). Effects of chronic hypoxia and endurance training on muscle capillarity in rats. *Pflugers Arch.* 419, 225–229. doi: 10.1007/BF00371099

Birot, O. J. G., Koulmann, N., Peinnequin, A., and Bigard, X. A. (2003). Exercise-induced expression of vascular endothelial growth factor mRNA in rat skeletal muscle is dependent on fibre type. *J. Physiol.* 552, 213–221. doi: 10.1113/jphysiol.2003.043026

Boegehold, M. A., and Johnson, P. C. (1988). Periarteriolar and tissue PO2 during sympathetic escape in skeletal muscle. *Am. J. Physiol.* 254, H929–H936. doi: 10.1152/ajpheart.1988.254.5.H929

Boos, C. J., Lamb, C. M., Midwinter, M., Mellor, A., Woods, D. R., Howley, M., et al. (2018). The effects of acute hypoxia on tissue oxygenation and circulating alarmins in healthy adults. *Physiol. Res.* 67, 935–943. doi: 10.33549/physiolres.933743

Booth, F. W., and Thomason, D. B. (1991). Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. *Physiol. Rev.* 71, 541–585. doi: 10.1152/physrev.1991.71.2.541

Breen, E. C., Johnson, E. C., Wagner, H., Tseng, H. M., Sung, L. A., and Wagner, P. D. (1996). Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J. Appl. Physiol.* 81, 355–361. doi: 10.1152/jappl.1996.81.1.355

Breen, E., Tang, K., Olfert, M., Knapp, A., and Wagner, P. (2008). Skeletal muscle capillarity during hypoxia: VEGF and its activation. *High Alt. Med. Biol.* 9, 158–166. doi: 10.1089/ham.2008.1010

Brinkmann, C., Metten, A., Scriba, P., Tagarakis, C. V. M., Wahl, P., Latsch, J., et al. (2017). Hypoxia and hyperoxia affect serum angiogenic regulators in T2DM men during cycling. *Int. J. Sports Med.* 38, 92–98. doi: 10.1055/s-0042-116823

Brocherie, F., Millet, G. P., D'Hulst, G., Van Thienen, R., Deldicque, L., and Girard, O. (2018). Repeated maximal-intensity hypoxic exercise superimposed to hypoxic residence boosts skeletal muscle transcriptional responses in elite team-sport athletes. *Acta Physiol.* 222 :e12851. doi: 10.1111/apha.12851

Burtscher, M., Pachinger, O., Ehrenbourg, I., Mitterbauer, G., Faulhaber, M., Pühringer, R., et al. (2004). Intermittent hypoxia increases exercise tolerance in elderly men with and without coronary artery disease. *Int. J. Cardiol.* 96, 247–254. doi: 10.1016/j.ijcard.2003.07.021

Camacho-Cardenosa, A., Camacho-Cardenosa, M., Brazo-Sayavera, J., Burtscher, M., Timón, R., and Olcina, G. (2018). Effects of high-intensity interval training under normobaric hypoxia on cardiometabolic risk markers in overweight/obese women. *High Alt. Med. Biol.* 19, 356–366. doi: 10.1089/ham.2018.0059

Camacho-Cardenosa, A., Camacho-Cardenosa, M., Brazo-Sayavera, J., Timón, R., González-Custodio, A., and Olcina, G. (2020). Repeated sprint in hypoxia as a time-metabolic efficient strategy to improve physical fitness of obese women. *Eur. J. Appl. Physiol.* 120, 1051–1061. doi: 10.1007/s00421-020-04344-2

Camacho-Cardenosa, A., Camacho-Cardenosa, M., Olcina, G., Timón, R., and Brazo-Sayavera, J. (2019). Detraining effect on overweight/obese women after high-intensity interval training in hypoxia. *Scand. J. Med. Sci. Sports* 29, 535–543. doi: 10.1111/sms.13380

Casey, D. P., and Joyner, M. J. (2011). Local control of skeletal muscle blood flow during exercise: influence of available oxygen. *J. Appl. Physiol.* 111, 1527–1538. doi: 10.1152/japplphysiol.00895.2011

Casey, D. P., and Joyner, M. J. (2012). Compensatory vasodilatation during hypoxic exercise: mechanisms responsible for matching oxygen supply to demand. *J. Physiol.* 590, 6321–6326. doi: 10.1113/jphysiol.2012.242396

Cassin, S., Gilbert, R. D., Bunnell, C. E., and Johnson, E. M. (1971). Capillary development during exposure to chronic hypoxia. *Am. J. Physiol.* 220, 448–451. doi: 10.1152/ajplegacy.1971.220.2.448

Chapman, R. F. (2013). The individual response to training and competition at altitude. *Br. J. Sports Med.* 47(Suppl. 1), i40–i44. doi: 10.1136/bjsports-2013-092837

Colburn, T. D., Hirai, D. M., Craig, J. C., Ferguson, S. K., Weber, R. E., Schulze, K. M., et al. (2020). Transcapillary PO2 gradients in contracting muscles across the fibre type and oxidative continuum. *J. Physiol.* 598, 3187–3202. doi: 10.1113/JP279608

Coppel, J., Hennis, P., Gilbert-Kawai, E., and Grocott, M. P. (2015). The physiological effects of hypobaric hypoxia versus normobaric hypoxia: a systematic review of crossover trials. *Extreme Physiol. Med.* 4:2. doi: 10.1186/s13728-014-0021-6

Croley, A. N., Zwetsloot, K. A., Westerkamp, L. M., Ryan, N. A., Pendergast, A. M., Hickner, R. C., et al. (2005). Lower capillarization, VEGF protein, and VEGF mRNA response to acute exercise in the vastus lateralis muscle of aged vs. young women. *J. Appl. Physiol.* 99, 1872–1879. doi: 10.1152/japplphysiol.00498.2005

D'Hulst, G., and Deldicque, L. (2017). Human skeletal muscle wasting in hypoxia: a matter of hypoxic dose? *J. Appl. Physiol.* 122, 406–408. doi: 10.1152/japplphysiol.00264.2016

D'Hulst, G., Ferri, A., Naslain, D., Bertrand, L., Horman, S., Francaux, M., et al. (2016). Fifteen days of 3,200 m simulated hypoxia marginally regulates markers for protein synthesis and degradation in human skeletal muscle. *Hypoxia* 4, 1–14. doi: 10.2147/HP.S101133

Daneshrad, Z., Novel-Chaté, V., Birot, O., Serrurier, B., Sanchez, H., Bigard, A. X., et al. (2001). Diet restriction plays an important role in the alterations of heart mitochondrial function following exposure of young rats to chronic hypoxia. *Pflugers Arch.* 442, 12–18. doi: 10.1007/s004240000461

Daneshrad, Z., Verdys, M., Birot, O., Troff, F., Bigard, A. X., and Rossi, A. (2003). Chronic hypoxia delays myocardial lactate dehydrogenase maturation in young rats. *Exp. Physiol.* 88, 405–413. doi: 10.1113/eph8802451

Debevec, T., and Millet, G. P. (2014). Discerning normobaric and hypobaric hypoxia: significance of exposure duration. *J. Appl. Physiol.* 116:1255. doi: 10.1152/japplphysiol.00873.2013

Delezie, J., and Handschin, C. (2018). Endocrine crosstalk between skeletal muscle and the brain. *Front. Neurol.* 9:698. doi: 10.3389/fneur.2018.00698

Desplanches, D., Hoppeler, H., Linossier, M. T., Denis, C., Claassen, H., Dormois, D., et al. (1993). Effects of training in normoxia and normobaric hypoxia on human muscle ultrastructure. *Pflugers Arch.* 425, 263–267. doi: 10.1007/BF00374176

Desplanches, D., Hoppeler, H., Tüscher, L., Mayet, M. H., Spielvogel, H., Ferretti, G., et al. (1996). Muscle tissue adaptations of high-altitude natives to training in chronic hypoxia or acute normoxia. *J. Appl. Physiol.* 81, 1946–1951. doi: 10.1152/jappl.1996.81.5.1946

Deveci, D., and Egginton, S. (2003). Cold exposure differentially stimulates angiogenesis in glycolytic and oxidative muscles of rats and hamsters. *Exp. Physiol.* 88, 741–746. doi: 10.1113/eph8802630

Deveci, D., Marshall, J. M., and Egginton, S. (2001). Relationship between capillary angiogenesis, fiber type, and fiber size in chronic systemic hypoxia. *Am. J. Physiol. Heart Circ. Physiol.* 281, H241–H252. doi: 10.1152/ajpheart.2001.281.1.H241

Deveci, D., Marshall, J. M., and Egginton, S. (2002). Chronic hypoxia induces prolonged angiogenesis in skeletal muscles of rat. *Exp. Physiol.* 87, 287–291. doi: 10.1113/eph8702377

Dinenno, F. A. (2016). Skeletal muscle vasodilation during systemic hypoxia in humans. *J. Appl. Physiol.* 120, 216–225. doi: 10.1152/japplphysiol.00256.2015

Ding, H., Liu, Q., Hua, M., Ding, M., Du, H., Zhang, W., et al. (2012). Associations between vascular endothelial growth factor gene polymorphisms and susceptibility to acute mountain sickness. *J. Int. Med. Res.* 40, 2135–2144. doi: 10.1177/030006051204000611

DiPasquale, D. M., Strangman, G. E., Harris, N. S., and Muza, S. R. (2016). Acute mountain sickness symptoms depend on normobaric versus hypobaric hypoxia. *BioMed Res. Int.* 2016:6245609. doi: 10.1155/2016/6245609

Domigan, C. K., Warren, C. M., Antanesian, V., Happel, K., Ziyad, S., Lee, S., et al. (2015). Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy. *J. Cell Sci.* 128, 2236–2248. doi: 10.1242/jcs.163774

Dorward, D. A., Roger Thompson, A. A., Kenneth Baillie, J., MacDougall, M., and Hirani, N. (2007). Change in plasma vascular endothelial growth factor during onset and recovery from acute mountain sickness. *Respir. Med.* 101, 587–594. doi: 10.1016/j.rmed.2006.06.014

Dunford, E. C., Leclair, E., Aiken, J., Mandel, E. R., Haas, T. L., Birot, O., et al. (2017). The effects of voluntary exercise and prazosin on capillary rarefaction and metabolism in streptozotocin-induced diabetic male rats. *J. Appl. Physiol.* 122, 492–502. doi: 10.1152/japplphysiol.00762.2016

Egginton, S. (2002). Temperature and angiogenesis: the possible role of mechanical factors in capillary growth. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 132, 773–787. doi: 10.1016/S1095-6433(02)00047-8

Egginton, S. (2009). Invited review: activity-induced angiogenesis. *Pflügers Arch.* 457, 963–977. doi: 10.1007/s00424-008-0563-9

Egginton, S. (2011). In vivo shear stress response. *Biochem. Soc. Trans.* 39, 1633–1638. doi: 10.1042/BST20110715

Egginton, S., and Birot, O. (2014). Angiogenesis: growth points. *Microcirculation* 21, 276–277. doi: 10.1111/micc.12131

Espinoza, J. R., Alvarez, G., León-Velarde, F., Ju Preciado, H. F., Macarlupu, J.-L., Rivera-Ch, M., et al. (2014). Vascular endothelial growth factor-A is associated with chronic mountain sickness in the Andean population. *High Alt. Med. Biol.* 15, 146–154. doi: 10.1089/ham.2013.1121

Fabel, K., Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N., et al. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur. J. Neurosci.* 18, 2803–2812. doi: 10.1111/j.1460-9568.2003.03041.x

Faiss, R., Girard, O., and Millet, G. P. (2013). Advancing hypoxic training in team sports: from intermittent hypoxic training to repeated sprint training in hypoxia: table 1. *Br. J. Sports Med.* 47, i45–i50. doi: 10.1136/bjsports-2013-092741

Favier, F. B., Britto, F. A., Freyssenet, D. G., Bigard, X. A., and Benoit, H. (2015). HIF-1-driven skeletal muscle adaptations to chronic hypoxia: molecular insights into muscle physiology. *Cell. Mol. Life Sci.* 72, 4681–4696. doi: 10.1007/s00018-015-2025-9

Fraisl, P., Mazzone, M., Schmidt, T., and Carmeliet, P. (2009). Regulation of angiogenesis by oxygen and metabolism. *Dev. Cell* 16, 167–179. doi: 10.1016/j.devcel.2009.01.003

Fujino, H., Kohzuki, H., Takeda, I., Kiyooka, T., Miyasaka, T., Mohri, S., et al. (2005). Regression of capillary network in atrophied soleus muscle induced by hindlimb unweighting. *J. Appl. Physiol.* 98, 1407–1413. doi: 10.1152/japplphysiol.00961.2004

Gardner, A. W., and Poehlman, E. T. (1995). Exercise rehabilitation programs for the treatment of claudication pain. A meta-analysis. *JAMA* 274, 975–980.

Gavin, T. P., and Wagner, P. D. (2001). Effect of short-term exercise training on angiogenic growth factor gene responses in rats. *J. Appl. Physiol.* 90, 1219–1226. doi: 10.1152/jappl.2001.90.4.1219

Gavin, T. P., Drew, J. L., Kubik, C. J., Pofahl, W. E., and Hickner, R. C. (2007). Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiol.* 191, 139–146. doi: 10.1111/j.1748-1716.2007.01723.x

Gavin, T. P., Westerkamp, L. M., and Zwetsloot, K. A. (2006). Soleus, plantaris and gastrocnemius VEGF mRNA responses to hypoxia and exercise are preserved in aged compared with young female C57BL/6 mice. *Acta Physiol.* 188, 113–121. doi: 10.1111/j.1748-1716.2006.01609.x

Gerber, H. P., Condorelli, F., Park, J., and Ferrara, N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J. Biol. Chem.* 272, 23659–23667. doi: 10.1074/jbc.272.38.23659

Gilbert-Kawai, E. T., Milledge, J. S., Grocott, M. P. W., and Martin, D. S. (2014). King of the mountains: tibetan and sherpa physiological adaptations for life at high altitude. *Physiology* 29, 388–402. doi: 10.1152/physiol.00018.2014

Girard, O., Amann, M., Aughey, R., Billaut, F., Bishop, D. J., Bourdon, P., et al. (2013). Position statement—altitude training for improving team-sport players' performance: current knowledge and unresolved issues. *Br. J. Sports Med.* 47, i8–i16. doi: 10.1136/bjsports-2013-093109

Girard, O., and Chalabi, H. (2013). Could altitude training benefit team-sport athletes? *Br. J. Sports Med.* 47, i4–i5. doi: 10.1136/bjsports-2013-092807

Girard, O., and Pluim, B. M. (2013). Improving team-sport player's physical performance with altitude training: from beliefs to scientific evidence. *Br. J. Sports Med.* 47, i2–i3. doi: 10.1136/bjsports-2013-093119

Girard, O., Brocherie, F., Goods, P. S. R., and Millet, G. P. (2020). An updated panorama of "living low-training high" altitude/hypoxic methods. *Front. Sports Act. Living* 2:26. doi: 10.3389/fspor.2020.00026

Girard, O., Koehle, M. S., MacInnis, M. J., Guenette, J. A., Koehle, M. S., Verges, S., et al. (2012). Comments on point:counterpoint: hypobaric hypoxia induces/does not induce different responses from normobaric hypoxia. *J. Appl. Physiol.* 112, 1788–1794. doi: 10.1152/japplphysiol.00356.2012

Gliemann, L., Buess, R., Nyberg, M., Hoppeler, H., Odriozola, A., Thaning, P., et al. (2015). Capillary growth, ultrastructure remodelling and exercise training in skeletal muscle of essential hypertensive patients. *Acta Physiol. Oxf. Engl.* 214, 210–220. doi: 10.1111/apha.12501

Gomarasca, M., Banfi, G., and Lombardi, G. (2020). Myokines: the endocrine coupling of skeletal muscle and bone. *Adv. Clin. Chem.* 94, 155–218. doi: 10.1016/bs.acc.2019.07.010

Gouzi, F., Préfaut, C., Abdellaoui, A., Roudier, E., de Rigal, P., Molinari, N., et al. (2013). Blunted muscle angiogenic training-response in COPD patients versus sedentary controls. *Eur. Respir. J.* 41, 806–814. doi: 10.1183/09031936.00053512

Green, H. J., Sutton, J. R., Cymerman, A., Young, P. M., and Houston, C. S. (1989). Operation everest II: adaptations in human skeletal muscle. *J. Appl. Physiol.* 66, 2454–2461. doi: 10.1152/jappl.1989.66.5.2454

Green, H. J., Sutton, J. R., Wolfel, E. E., Reeves, J. T., Butterfield, G. E., and Brooks, G. A. (1992). Altitude acclimatization and energy metabolic adaptations in skeletal muscle during exercise. *J. Appl. Physiol.* 73, 2701–2708. doi: 10.1152/jappl.1992.73.6.2701

Gunga, H.-C., Fries, D., Humpeler, E., Kirsch, K., Boldt, L.-E., Koralewski, E., et al. (2003). Austrian moderate altitude study (AMAS 2000) – fluid shifts, erythropoiesis, and angiogenesis in patients with metabolic syndrome at moderate altitude (≅1700 m). *Eur. J. Appl. Physiol.* 88, 497–505. doi: 10.1007/s00421-002-0734-x

Gunga, H.-C., Kirsch, K., Röcker, L., Behn, C., Koralewski, E., Davila, E. H., et al. (1999). Vascular endothelial growth factor in exercising humans under different environmental conditions. *Eur. J. Appl. Physiol.* 79, 484–490. doi: 10.1007/s004210050541

Gustafsson, T., Ameln, H., Fischer, H., Sundberg, C. J., Timmons, J. A., and Jansson, E. (2005). VEGF-A splice variants and related receptor expression in human skeletal muscle following submaximal exercise. *J. Appl. Physiol.* 98, 2137–2146. doi: 10.1152/japplphysiol.01402.2004

Gustafsson, T., and Kraus, W. E. (2001). Exercise-induced angiogenesis-related growth and transcription factors in skeletal muscle, and their modification in muscle pathology. *Front. Biosci.* 6:D75–D89. doi: 10.2741/gustafss

Gustafsson, T., Knutsson, A., Puntschart, A., Kaijser, L., Nordqvist, A.-C. S., Sundberg, C. J., et al. (2002). Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. *Pflugers Arch.* 444, 752–759. doi: 10.1007/s00424-002-0845-6

Haas, T. L., and Nwadozi, E. (2015). Regulation of skeletal muscle capillary growth in exercise and disease. *Appl. Physiol. Nutr. Metab.* 40, 1221–1232. doi: 10.1139/apnm-2015-0336

Hanaoka, M., Droma, Y., Naramoto, A., Honda, T., Kobayashi, T., and Kubo, K. (2003). Vascular endothelial growth factor in patients with high-altitude pulmonary edema. *J. Appl. Physiol.* 94, 1836–1840. doi: 10.1152/japplphysiol.00575.2002

Hellsten, Y., and Hoier, B. (2014). Capillary growth in human skeletal muscle: physiological factors and the balance between pro-angiogenic and angiostatic factors. *Biochem. Soc. Trans.* 42, 1616–1622. doi: 10.1042/BST20140197

Hepple, R. T. (2000). Skeletal muscle: microcirculatory adaptation to metabolic demand. *Med. Sci. Sports Exerc.* 32, 117–123. doi: 10.1097/00005768-200001000-00018

Hepple, R. T., Agey, P. J., Hazelwood, L., Szewczak, J. M., MacMillen, R. E., and Mathieu-Costello, O. (1998). Increased capillarity in leg muscle of finches living at altitude. *J. Appl. Physiol.* 85, 1871–1876. doi: 10.1152/jappl.1998.85.5.1871

Hirai, D. M., Colburn, T. D., Craig, J. C., Hotta, K., Kano, Y., Musch, T. I., et al. (2019). Skeletal muscle interstitial O2 pressures: bridging the gap between the capillary and myocyte. *Microcirculation* 26:e12497. doi: 10.1111/micc.12497

Hirai, D. M., Craig, J. C., Colburn, T. D., Eshima, H., Kano, Y., Sexton, W. L., et al. (2018). Skeletal muscle microvascular and interstitial PO2 from rest to contractions. *J. Physiol.* 596, 869–883. doi: 10.1113/JP275170

Hoier, B., Nordsborg, N., Andersen, S., Jensen, L., Nybo, L., Bangsbo, J., et al. (2012). Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training: angiogenic factors in human muscle. *J. Physiol.* 590, 595–606. doi: 10.1113/jphysiol.2011.216135

Hoier, B., Olsen, K., Hanskov, D. J. A., Jorgensen, M., Norup, L. R., and Hellsten, Y. (2020). Early time course of change in angiogenic proteins in human skeletal muscle and vascular cells with endurance training. *Scand. J. Med. Sci. Sports* 30, 1117–1131. doi: 10.1111/sms.13665

Hoppeler, H. (1999). Vascular growth in hypoxic skeletal muscle. *Adv. Exp. Med. Biol.* 474, 277–286. doi: 10.1007/978-1-4615-4711-2_21

Hoppeler, H., and Kayar, S. (1988). Capillarity and oxidative capacity of muscles. *Physiology* 3, 113–116. doi: 10.1152/physiologyonline.1988.3.3.113

Hoppeler, H., and Vogt, M. (2001). Muscle tissue adaptations to hypoxia. *J. Exp. Biol.* 204, 3133–3139.

Hoppeler, H., Hudlicka, O., and Uhlmann, E. (1987). Relationship between mitochondria and oxygen consumption in isolated cat muscles. *J. Physiol.* 385, 661–675. doi: 10.1113/jphysiol.1987.sp016513

Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S. R., et al. (1990a). Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int. J. Sports Med.* 11(Suppl. 1), S3–S9. doi: 10.1055/s-2007-1024846

Hoppeler, H., Howald, H., and Cerretelli, P. (1990b). Human muscle structure after exposure to extreme altitude. *Experientia* 46, 1185–1187. doi: 10.1007/BF01936933

Hoppeler, H., Lüthi, P., Claassen, H., Weibel, E. R., and Howald, H. (1973). The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. *Pflugers Arch.* 344, 217–232. doi: 10.1007/BF00588462

Hoppeler, H., Vogt, M., Weibel, E. R., and Flück, M. (2003). Response of skeletal muscle mitochondria to hypoxia. *Exp. Physiol.* 88, 109–119. doi: 10.1113/eph8802513

Horscroft, J. A., and Murray, A. J. (2014). Skeletal muscle energy metabolism in environmental hypoxia: climbing towards consensus. *Extreme Physiol. Med.* 3:19. doi: 10.1186/2046-7648-3-19

Horscroft, J. A., Kotwica, A. O., Laner, V., West, J. A., Hennis, P. J., Levett, D. Z. H., et al. (2017). Metabolic basis to Sherpa altitude adaptation. *Proc. Natl. Acad. Sci. U.S.A.* 114, 6382–6387. doi: 10.1073/pnas.1700527114

Howlett, R. A., Gonzalez, N. C., Wagner, H. E., Fu, Z., Britton, S. L., Koch, L. G., et al. (2003). Selected contribution: skeletal muscle capillarity and enzyme activity in rats selectively bred for running endurance. *J. Appl. Physiol.* 94, 1682–1688. doi: 10.1152/japplphysiol.00556.2002

Hudlicka, O. (2011). Microcirculation in skeletal muscle. *Muscles Ligaments Tendons J.* 1, 3–11.

Hudlicka, O., and Brown, M. D. (2009). Adaptation of skeletal muscle microvasculature to increased or decreased blood flow: role of shear stress, nitric oxide and vascular endothelial growth factor. *J. Vasc. Res.* 46, 504–512. doi: 10.1159/000226127

Hudlicka, O., Brown, M. D., Egginton, S., and Dawson, J. M. (1994). Effect of long-term electrical stimulation on vascular supply and fatigue in chronically ischemic muscles. *J. Appl. Physiol.* 77, 1317–1324. doi: 10.1152/jappl.1994.77.3.1317

Hudlicka, O., Brown, M., and Egginton, S. (1992). Angiogenesis in skeletal and cardiac muscle. *Physiol. Rev.* 72, 369–417. doi: 10.1152/physrev.1992.72.2.369

Hudlicka, O., Hoppeler, H., and Uhlmann, E. (1987). Relationship between the size of the capillary bed and oxidative capacity in various cat skeletal muscles. *Pflugers Arch.* 410, 369–375. doi: 10.1007/BF00586513

Huez, I., Créancier, L., Audigier, S., Gensac, M. C., Prats, A. C., and Prats, H. (1998). Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. *Mol. Cell. Biol.* 18, 6178–6190. doi: 10.1128/MCB.18.11.6178

Hutter, J., Habler, O., Kleen, M., Tiede, M., Podtschaske, A., Kemming, G., et al. (1999). Effect of acute normovolemic hemodilution on distribution of blood flow and tissue oxygenation in dog skeletal muscle. *J. Appl. Physiol.* 86, 860–866. doi: 10.1152/jappl.1999.86.3.860

Jackson, C. G., Sillau, A. H., and Banchero, N. (1987). Fiber composition and capillarity in growing guinea pigs acclimated to cold and cold plus hypoxia. *Proc. Soc. Exp. Biol. Med.* 185, 101–106. doi: 10.3181/00379727-185-42524

Johnson, P. C., Vandegriff, K., Tsai, A. G., and Intaglietta, M. (2005). Effect of acute hypoxia on microcirculatory and tissue oxygen levels in rat cremaster muscle. *J. Appl. Physiol.* 98, 1177–1184. doi: 10.1152/japplphysiol.00591.2004

Joyner, M. J., and Casey, D. P. (2014). Muscle blood flow, hypoxia, and hypoperfusion. *J. Appl. Physiol.* 116, 852–857. doi: 10.1152/japplphysiol.00620.2013

Jung, W.-S., Kim, S.-W., Kim, J.-W., and Park, H.-Y. (2021). Resistance training in hypoxia as a new therapeutic modality for sarcopenia—a narrative review. *Life* 11:106. doi: 10.3390/life11020106

Kaiser, R., Frantz, C., Bals, R., and Wilkens, H. (2016). The role of circulating thrombospondin-1 in patients with precapillary pulmonary hypertension. *Respir. Res.* 17:96. doi: 10.1186/s12931-016-0412-x

Kasai, N., Kojima, C., Sumi, D., Ikutomo, A., and Goto, K. (2019). Inflammatory, oxidative stress, and angiogenic growth factor responses to repeated-sprint exercise in hypoxia. *Front. Physiol.* 10:844. doi: 10.3389/fphys.2019.00844

Kasperska, A., and Zembron-Lacny, A. (2020). The effect of intermittent hypoxic exposure on erythropoietic response and hematological variables in elite athletes. *Physiol. Res.* 69, 283–290. doi: 10.33549/physiolres.934316

Kayser, B., Hoppeler, H., Claassen, H., and Cerretelli, P. (1991). Muscle structure and performance capacity of Himalayan Sherpas. *J. Appl. Physiol.* 70, 1938–1942. doi: 10.1152/jappl.1991.70.5.1938

Kayser, B., Hoppeler, H., Desplanches, D., Marconi, C., Broers, B., and Cerretelli, P. (1996). Muscle ultrastructure and biochemistry of lowland Tibetans. *J. Appl. Physiol.* 81, 419–425. doi: 10.1152/jappl.1996.81.1.419

Ke, Q., and Costa, M. (2006). Hypoxia-inducible factor-1 (HIF-1). *Mol. Pharmacol.* 70, 1469–1480. doi: 10.1124/mol.106.027029

Kim, S., Choi, J.-Y., Moon, S., Park, D.-H., Kwak, H.-B., and Kang, J.-H. (2019). Roles of myokines in exercise-induced improvement of neuropsychiatric function. *Pflugers Arch.* 471, 491–505. doi: 10.1007/s00424-019-02253-8

Kindig, C. A., Howlett, R. A., and Hogan, M. C. (2003). Effect of extracellular P_{O_2} on the fall in intracellular P_{O_2} in contracting single myocytes. *J. Appl. Physiol.* 94, 1964–1970. doi: 10.1152/japplphysiol.00893.2002

Kishlyansky, M., Vojnovic, J., Roudier, E., Gineste, C., Decary, S., Forn, P., et al. (2010). Striated muscle angio-adaptation requires changes in Vasohibin-1 expression pattern. *Biochem. Biophys. Res. Commun.* 399, 359–364. doi: 10.1016/j.bbrc.2010.07.076

Kissane, R. W. P., Al-Shammari, A. A., and Egginton, S. (2021). The importance of capillary distribution in supporting muscle function, building on Krogh's seminal ideas. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 254:110889. doi: 10.1016/j.cbpa.2020.110889

Kivelä, R., Silvennoinen, M., Lehti, M., Jalava, S., Vihko, V., and Kainulainen, H. (2008). Exercise-induced expression of angiogenic growth factors in skeletal muscle and in capillaries of healthy and diabetic mice. *Cardiovasc. Diabetol.* 7:13. doi: 10.1186/1475-2840-7-13

Kivelä, R., Silvennoinen, M., Touvra, A.-M., Lehti, T. M., Kainulainen, H., and Vihko, V. (2006). Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle. *FASEB J.* 20, 1570–1572. doi: 10.1096/fj.05-4780fje

Koester-Hegmann, C., Bengoetxea, H., Kosenkov, D., Thiersch, M., Haider, T., Gassmann, M., et al. (2019). High-altitude cognitive impairment is prevented by enriched environment including exercise via VEGF signaling. *Front. Cell. Neurosci.* 12:532. doi: 10.3389/fncel.2018.00532

Kong, Z., Shi, Q., Nie, J., Tong, T. K., Song, L., Yi, L., et al. (2017). High-intensity interval training in normobaric hypoxia improves cardiorespiratory fitness in overweight Chinese young women. *Front. Physiol.* 8:175. doi: 10.3389/fphys.2017.00175

Kraus, R. M., Stallings, H. W., Yeager, R. C., and Gavin, T. P. (2004). Circulating plasma VEGF response to exercise in sedentary and endurance-trained men. *J. Appl. Physiol.* 96, 1445–1450. doi: 10.1152/japplphysiol.01031.2003

Krogh, A. (1919a). The number and distribution of capillaries in muscles with calculations of the oxygen pressure head necessary for supplying the tissue. *J. Physiol.* 52, 409–415. doi: 10.1113/jphysiol.1919.sp001839

Krogh, A. (1919b). The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *J. Physiol.* 52, 391–408. doi: 10.1113/jphysiol.1919.sp001838

Krogh, A. (1919c). The supply of oxygen to the tissues and the regulation of the capillary circulation. *J. Physiol.* 52, 457–474. doi: 10.1113/jphysiol.1919.sp001844

Lee, S., Chen, T. T., Barber, C. L., Jordan, M. C., Murdock, J., Desai, S., et al. (2007). Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 130, 691–703. doi: 10.1016/j.cell.2007.06.054

Levett, D. Z., Radford, E. J., Menassa, D. A., Graber, E. F., Morash, A. J., Hoppeler, H., et al. (2012). Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *FASEB J.* 26, 1431–1441. doi: 10.1096/fj.11-197772

Levine, B. D. (2002). Intermittent hypoxic training: fact and fancy. *High Alt. Med. Biol.* 3, 177–193. doi: 10.1089/15270290260131911

Levine, B. D., and Stray-Gundersen, J. (2006). "Dose-response of altitude training: how much altitude is enough?," in *Hypoxia and Exercise*, eds R. C. Roach, P. D. Wagner, and P. H. Hackett (Boston, MA: Springer US), 233–247. doi: 10.1007/978-0-387-34817-9_20

Lindholm, M. E., and Rundqvist, H. (2016). Skeletal muscle hypoxia-inducible factor-1 and exercise: skeletal muscle hypoxia-inducible factor-1 and exercise. *Exp. Physiol.* 101, 28–32. doi: 10.1113/EP085318

Lindholm, M. E., Fischer, H., Poellinger, L., Johnson, R. S., Gustafsson, T., Sundberg, C. J., et al. (2014). Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R248–R255. doi: 10.1152/ajpregu.00036.2013

Liu, G., Mac Gabhann, F., and Popel, A. S. (2012). Effects of fiber type and size on the heterogeneity of oxygen distribution in exercising skeletal muscle. *PLoS One* 7:e44375. doi: 10.1371/journal.pone.0044375

Lombard, J. H., Frisbee, J. C., Greene, A. S., Hudetz, A. G., Roman, R. J., and Tonellato, P. J. (2000). Microvascular flow and tissue PO(2) in skeletal muscle of chronic reduced renal mass hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* 279, H2295–H2302. doi: 10.1152/ajpheart.2000.279.5.H2295

Lui, M. A., Mahalingam, S., Patel, P., Connaty, A. D., Ivy, C. M., Cheviron, Z. A., et al. (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 308, R779–R791. doi: 10.1152/ajpregu.00362.2014

Lundby, C. (2004). Acclimatization to 4100 m does not change capillary density or mRNA expression of potential angiogenesis regulatory factors in human skeletal muscle. *J. Exp. Biol.* 207, 3865–3871. doi: 10.1242/jeb.01225

Lundby, C., Calbet, J. A. L., and Robach, P. (2009). The response of human skeletal muscle tissue to hypoxia. *Cell. Mol. Life Sci.* 66, 3615–3623. doi: 10.1007/s00018-009-0146-8

Lundby, C., Gassmann, M., and Pilegaard, H. (2006). Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. *Eur. J. Appl. Physiol.* 96, 363–369. doi: 10.1007/s00421-005-0085-5

Lundby, C., Millet, G. P., Calbet, J. A., Bärtsch, P., and Subudhi, A. W. (2012). Does "altitude training" increase exercise performance in elite athletes? *Br. J. Sports Med.* 46, 792–795. doi: 10.1136/bjsports-2012-091231

MacDougall, J. D., Green, H. J., Sutton, J. R., Coates, G., Cymerman, A., Young, P., et al. (1991). Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta Physiol. Scand.* 142, 421–427. doi: 10.1111/j.1748-1716.1991.tb09176.x

Malek, M. H., and Olfert, I. M. (2009). Global deletion of thrombospondin-1 increases cardiac and skeletal muscle capillarity and exercise capacity in mice. *Exp. Physiol.* 94, 749–760. doi: 10.1113/expphysiol.2008.045989

Malek, M. H., Olfert, I. M., and Esposito, F. (2010). Detraining losses of skeletal muscle capillarization are associated with vascular endothelial growth factor protein expression in rats. *Exp. Physiol.* 95, 359–368. doi: 10.1113/expphysiol.2009.050369

Mathieu-Costello, O. (1994). "Morphometry of the size of the capillary-to-fiber interface in muscles," in *Oxygen Transport to Tissue XV, Advances in Experimental Medicine and Biology*, eds P. Vaupel, R. Zander, and D. F. Bruley (Boston, MA: Springer US), 661–668. doi: 10.1007/978-1-4615-2468-7_87

Mathieu-Costello, O., and Agey, P. J. (1997). Chronic hypoxia affects capillary density and geometry in pigeon pectoralis muscle. *Respir. Physiol.* 109, 39–52. doi: 10.1016/S0034-5687(97)84028-5

McDermott, M. M., Ades, P., Guralnik, J. M., Dyer, A., Ferrucci, L., Liu, K., et al. (2009). Treadmill exercise and resistance training in patients with peripheral arterial disease with and without intermittent claudication: a randomized controlled trial. *JAMA* 301, 165–174. doi: 10.1001/jama.2008.962

Milkiewicz, M., Brown, M. D., Egginton, S., and Hudlicka, O. (2001). Association between shear stress, angiogenesis, and VEGF in skeletal muscles in vivo. *Microcirculation* 8, 229–241. doi: 10.1038/sj/mn/7800074

Milkiewicz, M., Doyle, J. L., Fudalewski, T., Ispanovic, E., Aghasi, M., and Haas, T. L. (2007). HIF-1α and HIF-2α play a central role in stretch-induced but not shear-stress-induced angiogenesis in rat skeletal muscle: HIF-1α and HIF-2α in stretch-induced angiogenesis. *J. Physiol.* 583, 753–766. doi: 10.1113/jphysiol.2007.136325

Milkiewicz, M., Roudier, E., Doyle, J. L., Trifonova, A., Birot, O., and Haas, T. L. (2011). Identification of a mechanism underlying regulation of the anti-angiogenic forkhead transcription factor FoxO1 in cultured endothelial cells and ischemic muscle. *Am. J. Pathol.* 178, 935–944. doi: 10.1016/j.ajpath.2010.10.042

Miller, D. L., Dibbens, J. A., Damert, A., Risau, W., Vadas, M. A., and Goodall, G. J. (1998). The vascular endothelial growth factor mRNA contains an internal ribosome entry site. *FEBS Lett.* 434, 417–420. doi: 10.1016/s0014-5793(98)01025-4

Millet, G. P., Debevec, T., Brocherie, F., Girard, O., Pialoux, V., Wüst, R. C. I., et al. (2017). Commentaries on viewpoint: human skeletal muscle wasting in hypoxia: a matter of hypoxic dose? *J. Appl. Physiol.* 122, 409–411. doi: 10.1152/japplphysiol.01084.2016

Millet, G. P., Debevec, T., Brocherie, F., Malatesta, D., and Girard, O. (2016). Therapeutic use of exercising in hypoxia: promises and limitations. *Front. Physiol.* 7:224. doi: 10.3389/fphys.2016.00224

Millet, G. P., Faiss, R., and Pialoux, V. (2012a). Last word on point: counterpoint: hypobaric hypoxia induces different responses from normobaric hypoxia. *J. Appl. Physiol.* 112:1795. doi: 10.1152/japplphysiol.00338.2012

Millet, G. P., Faiss, R., and Pialoux, V. (2012b). Point: Hypobaric hypoxia induces different physiological responses from normobaric hypoxia. *J. Appl. Physiol.* 112, 1783–1784. doi: 10.1152/japplphysiol.00067.2012

Millet, G. P., Faiss, R., and Pialoux, V. (2013). Evidence for differences between hypobaric and normobaric hypoxia is conclusive. *Exerc. Sport Sci. Rev.* 41:133. doi: 10.1097/JES.0b013e318271a5e1

Millet, G. P., Roels, B., Schmitt, L., Woorons, X., and Richalet, J. P. (2010). Combining hypoxic methods for peak performance. *Sports Med.* 40, 1–25. doi: 10.2165/11317920-000000000-00000

Mizuno, M., Juel, C., Bro-Rasmussen, T., Mygind, E., Schibye, B., Rasmussen, B., et al. (1990). Limb skeletal muscle adaptation in athletes after training at altitude. *J. Appl. Physiol.* 68, 496–502. doi: 10.1152/jappl.1990.68.2.496

Mizuno, M., Savard, G. K., Areskog, N.-H., Lundby, C., and Saltin, B. (2008). Skeletal muscle adaptations to prolonged exposure to extreme altitude: a role of physical activity? *High Alt. Med. Biol.* 9, 311–317. doi: 10.1089/ham.2008.1009

Molé, P. A., Chung, Y., Tran, T. K., Sailasuta, N., Hurd, R., and Jue, T. (1999). Myoglobin desaturation with exercise intensity in human gastrocnemius muscle. *Am. J. Physiol.* 277, R173–R180. doi: 10.1152/ajpregu.1999.277.1.R173

Morfoisse, F., Renaud, E., Hantelys, F., Prats, A.-C., and Garmy-Susini, B. (2015). Role of hypoxia and vascular endothelial growth factors in lymphangiogenesis. *Mol. Cell. Oncol.* 2:e1024821. doi: 10.1080/23723556.2015.1024821

Morici, G., Bonanno, A., Licciardi, A., Valli, G., Passino, C., Bonardi, D., et al. (2013). Plasma leptin and vascular endothelial growth factor (VEGF) in normal subjects at high altitude (5050 m). *Arch. Physiol. Biochem.* 119, 219–224. doi: 10.3109/13813455.2013.814679

Mounier, R., and Brugniaux, J. V. (2012a). Counterpoint: hypobaric hypoxia does not induce different responses from normobaric hypoxia. *J. Appl. Physiol.* 112, 1784–1786. doi: 10.1152/japplphysiol.00067.2012a

Mounier, R., and Brugniaux, J. V. (2012b). Last word on counterpoint: hypobaric hypoxia does not induce different physiological responses from normobaric hypoxia. *J. Appl. Physiol.* 112:1796. doi: 10.1152/japplphysiol.00355.2012

Murray, A. J., and Horscroft, J. A. (2016). Mitochondrial function at extreme high altitude: mitochondrial function at extreme high altitude. *J. Physiol.* 594, 1137–1149. doi: 10.1113/JP270079

Nagahisa, H., Mukai, K., Ohmura, H., Takahashi, T., and Miyata, H. (2016). Effect of high-intensity training in normobaric hypoxia on thoroughbred skeletal muscle. *Oxid. Med. Cell. Longev.* 2016:1535367. doi: 10.1155/2016/1535367

Nilles, E., Sayward, H., and D'Onofrio, G. (2009). Vascular endothelial growth factor and acute mountain sickness. *J. Emerg. Trauma Shock* 2, 6–9. doi: 10.4103/0974-2700.44675

Ochoa, C. D., Yu, L., Al-Ansari, E., Hales, C. A., and Quinn, D. A. (2010). Thrombospondin-1 null mice are resistant to hypoxia-induced pulmonary hypertension. *J. Cardiothorac. Surg.* 5:32. doi: 10.1186/1749-8090-5-32

Oelz, O., Howald, H., Di Prampero, P. E., Hoppeler, H., Claassen, H., Jenni, R., et al. (1986). Physiological profile of world-class high-altitude climbers. *J. Appl. Physiol.* 60, 1734–1742. doi: 10.1152/jappl.1986.60.5.1734

Olenich, S. A., Audet, G. N., Roberts, K. A., and Olfert, I. M. (2014). Effects of detraining on the temporal expression of positive and negative angioregulatory proteins in skeletal muscle of mice. *J. Physiol.* 592, 3325–3338. doi: 10.1113/jphysiol.2014.271213

Olfert, I. M. (2016). Physiological capillary regression is not dependent on reducing VEGF expression. *Microcirculation* 23, 146–156. doi: 10.1111/micc.12263

Olfert, I. M., and Birot, O. (2011). Importance of anti-angiogenic factors in the regulation of skeletal muscle angiogenesis. *Microcirculation* 18, 316–330. doi: 10.1111/j.1549-8719.2011.00092.x

Olfert, I. M., Baum, O., Hellsten, Y., and Egginton, S. (2015). Advances and challenges in skeletal muscle angiogenesis. *Am. J. Physiol. Heart Circ. Physiol.* 310, H326–H336. doi: 10.1152/ajpheart.00635.2015

Olfert, I. M., Breen, E. C., Gavin, T. P., and Wagner, P. D. (2006). Temporal thrombospondin-1 mRNA response in skeletal muscle exposed to acute and chronic exercise. *Growth Factors* 24, 253–259. doi: 10.1080/08977190601000111

Olfert, I. M., Breen, E. C., Mathieu-Costello, O., and Wagner, P. D. (2001). Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. *J. Appl. Physiol.* 91, 1176–1184. doi: 10.1152/jappl.2001.91.3.1176

Olfert, I. M., Howlett, R. A., Tang, K., Dalton, N. D., Gu, Y., Peterson, K. L., et al. (2009). Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. *J. Physiol.* 587, 1755–1767. doi: 10.1113/jphysiol.2008.164384

Olfert, I. M., Howlett, R. A., Wagner, P. D., and Breen, E. C. (2010). Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299, R1059–R1067. doi: 10.1152/ajpregu.00347.2010

Oltmanns, K. M., Gehring, H., Rudolf, S., Schultes, B., Hackenberg, C., Schweiger, U., et al. (2006). Acute hypoxia decreases plasma VEGF concentration in healthy humans. *Am. J. Physiol. Endocrinol. Metab.* 290, E434–E439. doi: 10.1152/ajpendo.00508.2004

Osera, C., Martindale, J. L., Amadio, M., Kim, J., Yang, X., Moad, C. A., et al. (2015). Induction of VEGFA mRNA translation by CoCl2 mediated by HuR. *RNA Biol.* 12, 1121–1130. doi: 10.1080/15476286.2015.1085276

Ozawa, K., Kondo, T., Hori, O., Kitao, Y., Stern, D. M., Eisenmenger, W., et al. (2001a). Expression of the oxygen-regulated protein ORP150 accelerates wound healing by modulating intracellular VEGF transport. *J. Clin. Invest.* 108, 41–50. doi: 10.1172/JCI11772

Ozawa, K., Tsukamoto, Y., Hori, O., Kitao, Y., Yanagi, H., Stern, D. M., et al. (2001b). Regulation of tumor angiogenesis by oxygen-regulated protein 150, an inducible endoplasmic reticulum chaperone. *Cancer Res.* 61, 4206–4213.

Panisello, P., Torrella, J. R., Esteva, S., Pagés, T., and Viscor, G. (2008). Capillary supply, fibre types and fibre morphometry in rat tibialis anterior and diaphragm muscles after intermittent exposure to hypobaric hypoxia. *Eur. J. Appl. Physiol.* 103, 203–213. doi: 10.1007/s00421-008-0691-0

Patitucci, M., Lugrin, D., and Pagès, G. (2009). Angiogenic/lymphangiogenic factors and adaptation to extreme altitudes during an expedition to Mount Everest. *Acta Physiol.* 196, 259–265. doi: 10.1111/j.1748-1716.2008.01915.x

Phelan, M. W., Forman, L. W., Perrine, S. P., and Faller, D. V. (1998). Hypoxia increases thrombospondin-1 transcript and protein in cultured endothelial cells. *J. Lab. Clin. Med.* 132, 519–529. doi: 10.1016/s0022-2143(98)90131-7

Poole, D. C., and Mathieu-Costello, O. (1989). Skeletal muscle capillary geometry: adaptation to chronic hypoxia. *Respir. Physiol.* 77, 21–29. doi: 10.1016/0034-5687(89)90026-1

Poole, D. C., and Mathieu-Costello, O. (1996). Relationship between fiber capillarization and mitochondrial volume density in control and trained rat soleus and plantaris muscles. *Microcirculation* 3, 175–186. doi: 10.3109/10739689609148286

Poole, D. C., Kano, Y., Koga, S., and Musch, T. I. (2021). August Krogh: muscle capillary function and oxygen delivery. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 253:110852. doi: 10.1016/j.cbpa.2020.110852

Poole, D. C., Pittman, R. N., Musch, T. I., and Østergaard, L. (2020). August Krogh's theory of muscle microvascular control and oxygen delivery: a paradigm shift based on new data. *J. Physiol.* 598, 4473–4507. doi: 10.1113/JP279223

Pramsohler, S., Burtscher, M., Faulhaber, M., Gatterer, H., Rausch, L., Eliasson, A., et al. (2017). Endurance training in normobaric hypoxia imposes less physical stress for geriatric rehabilitation. *Front. Physiol.* 8:514. doi: 10.3389/fphys.2017.00514

Prewitt, R. L., and Johnson, P. C. (1976). The effect of oxygen on arteriolar red cell velocity and capillary density in the rat cremaster muscle. *Microvasc. Res.* 12, 59–70. doi: 10.1016/0026-2862(76)90007-8

Ramos-Campo, D. J., Girard, O., Pérez, A., and Rubio-Arias, J. Á (2019). Additive stress of normobaric hypoxic conditioning to improve body mass loss and cardiometabolic markers in individuals with overweight or obesity: a systematic review and meta-analysis. *Physiol. Behav.* 207, 28–40. doi: 10.1016/j.physbeh.2019.04.027

Rich, B., Scadeng, M., Yamaguchi, M., Wagner, P. D., and Breen, E. C. (2017). Skeletal myofiber vascular endothelial growth factor is required for the exercise training-induced increase in dentate gyrus neuronal precursor cells. *J. Physiol.* 595, 5931–5943. doi: 10.1113/JP273994

Richalet, J.-P. (2020a). CrossTalk opposing view: barometric pressure, independent of P O 2, is not the forgotten parameter in altitude physiology and mountain medicine. *J. Physiol.* 598, 897–899. doi: 10.1113/JP279160

Richalet, J.-P. (2020b). Rebuttal from Jean-Paul Richalet. *J. Physiol.* 598:903. doi: 10.1113/JP279426

Richard, N. A., Sahota, I. S., Widmer, N., Ferguson, S., Sheel, A. W., and Koehle, M. S. (2014). Acute mountain sickness, chemosensitivity, and cardiorespiratory responses in humans exposed to hypobaric and normobaric hypoxia. *J. Appl. Physiol.* 116, 945–952. doi: 10.1152/japplphysiol.00319.2013

Richardson, R. S. (2000). Intracellular Po2 and bioenergetic measurements in skeletal muscle: the role of exercise paradigm. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278, R1111–R1113. doi: 10.1152/ajpregu.2000.278.4.R1111

Richardson, R. S., Duteil, S., Wary, C., Wray, D. W., Hoff, J., and Carlier, P. G. (2006). Human skeletal muscle intracellular oxygenation: the impact of ambient oxygen availability: muscle oxygenation. *J. Physiol.* 571, 415–424. doi: 10.1113/jphysiol.2005.102327

Richardson, R. S., Noyszewski, E. A., Kendrick, K. F., Leigh, J. S., and Wagner, P. D. (1995). Myoglobin O2 desaturation during exercise. evidence of limited O2 transport. *J. Clin. Invest.* 96, 1916–1926. doi: 10.1172/JCI118237

Richmond, K. N., Burnite, S., and Lynch, R. M. (1997). Oxygen sensitivity of mitochondrial metabolic state in isolated skeletal and cardiac myocytes. *Am. J. Physiol. Cell Physiol.* 273, C1613–C1622. doi: 10.1152/ajpcell.1997.273.5.C1613

Richmond, K. N., Shonat, R. D., Lynch, R. M., and Johnson, P. C. (1999). Critical PO(2) of skeletal muscle in vivo. *Am. J. Physiol.* 277, H1831–H1840. doi: 10.1152/ajpheart.1999.277.5.H1831

Rogers, N. M., Sharifi-Sanjani, M., Yao, M., Ghimire, K., Bienes-Martinez, R., Mutchler, S. M., et al. (2017). TSP1-CD47 signaling is upregulated in clinical pulmonary hypertension and contributes to pulmonary arterial vasculopathy and dysfunction. *Cardiovasc. Res.* 113, 15–29. doi: 10.1093/cvr/cvw218

Roudier, E., Chapados, N., Decary, S., Gineste, C., Le Bel, C., Lavoie, J.-M., et al. (2009). Angiomotin p80/p130 ratio: a new indicator of exercise-induced

angiogenic activity in skeletal muscles from obese and non-obese rats? *J. Physiol.* 587, 4105–4119. doi: 10.1113/jphysiol.2009.175554

Roudier, E., Forn, P., Perry, M. E., and Birot, O. (2012). Murine double minute-2 expression is required for capillary maintenance and exercise-induced angiogenesis in skeletal muscle. *FASEB J.* 26, 4530–4539. doi: 10.1096/fj.12-212720

Roudier, E., Gineste, C., Wazna, A., Dehghan, K., Desplanches, D., and Birot, O. (2010). Angio-adaptation in unloaded skeletal muscle: new insights into an early and muscle type-specific dynamic process. *J. Physiol.* 588, 4579–4591. doi: 10.1113/jphysiol.2010.193243

Roudier, E., Milkiewicz, M., Birot, O., Slopack, D., Montelius, A., Gustafsson, T., et al. (2013). Endothelial FoxO1 is an intrinsic regulator of thrombospondin 1 expression that restrains angiogenesis in ischemic muscle. *Angiogenesis* 16, 759–772. doi: 10.1007/s10456-013-9353-x

Schnyder, S., and Handschin, C. (2015). Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise. *Bone* 80, 115–125. doi: 10.1016/j.bone.2015.02.008

Schobersberger, W., Hobisch-Hagen, P., Fries, D., Wiedermann, F., Rieder-Scharinger, J., Villiger, B., et al. (2000). Increase in immune activation, vascular endothelial growth factor and erythropoietin after an ultramarathon run at moderate altitude. *Immunobiology* 201, 611–620. doi: 10.1016/S0171-2985(00)80078-9

Schommer, K., Wiesegart, N., Dehnert, C., Mairbäurl, H., and Bärtsch, P. (2011). No correlation between plasma levels of vascular endothelial growth factor or its soluble receptor and acute mountain sickness. *High Alt. Med. Biol.* 12, 323–327. doi: 10.1089/ham.2011.1020

Scott, G. R., Elogio, T. S., Lui, M. A., Storz, J. F., and Cheviron, Z. A. (2015). Adaptive modifications of muscle phenotype in high-altitude deer mice are associated with evolved changes in gene regulation. *Mol. Biol. Evol.* 32, 1962–1976. doi: 10.1093/molbev/msv076

Semenza, G. L. (2001). Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance. *J. Clin. Invest.* 108, 39–40. doi: 10.1172/JCI13374

Semenza, G. L., and Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.* 12, 5447–5454. doi: 10.1128/mcb.12.12.5447

Sillau, A. H., and Banchero, N. (1977). Effects of hypoxia on capillary density and fiber composition in rat skeletal muscle. *Pflugers Arch.* 370, 227–232. doi: 10.1007/BF00585531

Sillau, A. H., Aquin, L., Bui, M. V., and Banchero, N. (1980a). Chronic hypoxia does not affect Guinea pig skeletal muscle capillarity. *Pflügers Arch.* 386, 39–45. doi: 10.1007/BF00584185

Sillau, A. H., Aquin, L., Lechner, A. J., Bui, M. V., and Banchero, N. (1980b). Increased capillary supply in skeletal muscle of guinea pigs acclimated to cold. *Respir. Physiol.* 42, 233–245. doi: 10.1016/0034-5687(80)90117-6

Slopack, D., Roudier, E., Liu, S. T. K., Nwadozi, E., Birot, O., and Haas, T. L. (2014). Forkhead BoxO transcription factors restrain exercise-induced angiogenesis. *J. Physiol.* 592, 4069–4082. doi: 10.1113/jphysiol.2014.275867

Snyder, G. K., Farrelly, C., and Coelho, J. R. (1992). Adaptations in skeletal muscle capillarity following changes in oxygen supply and changes in oxygen demands. *Eur. J. Appl. Physiol.* 65, 158–163. doi: 10.1007/BF00705074

Stein, I., Itin, A., Einat, P., Skaliter, R., Grossman, Z., and Keshet, E. (1998). Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol. Cell. Biol.* 18, 3112–3119. doi: 10.1128/MCB.18.6.3112

Stroka, D. M., Burkhardt, T., Desbaillets, I., Wenger, R. H., Neil, D. A. H., Bauer, C., et al. (2001). HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J.* 15, 2445–2453. doi: 10.1096/fj.01-0125com

Swenson, E. R., and Bärtsch, P. (2013). *High Altitude: Human Adaptation to Hypoxia*. Berlin: Springer Science & Business Media.

Tang, K., Breen, E. C., and Wagner, P. D. (2002). Hu protein R-mediated posttranscriptional regulation of VEGF expression in rat gastrocnemius muscle. *Am. J. Physiol. Heart Circ. Physiol.* 283, H1497–H1504. doi: 10.1152/ajpheart.00813.2001

Tang, K., Breen, E. C., Gerber, H.-P., Ferrara, N. M. A., and Wagner, P. D. (2004). Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. *Physiol. Genomics* 18, 63–69. doi: 10.1152/physiolgenomics.00023.2004

Terrados, N., Melichna, J., Sylvén, C., Jansson, E., and Kaijser, L. (1988). Effects of training at simulated altitude on performance and muscle metabolic capacity in competitive road cyclists. *Eur. J. Appl. Physiol.* 57, 203–209. doi: 10.1007/BF00640664

Tissot van Patot, M. C., Leadbetter, G., Keyes, L. E., Bendrick-Peart, J., Beckey, V. E., Christians, U., et al. (2005). Greater free plasma VEGF and lower soluble VEGF receptor-1 in acute mountain sickness. *J. Appl. Physiol.* 98, 1626–1629. doi: 10.1152/japplphysiol.00589.2004

van Ekeren, G. J., Sengers, R. C., and Stadhouders, A. M. (1992). Changes in volume densities and distribution of mitochondria in rat skeletal muscle after chronic hypoxia. *Int. J. Exp. Pathol.* 73, 51–60.

Verges, S., Chacaroun, S., Godin-Ribuot, D., and Baillieul, S. (2015). Hypoxic conditioning as a new therapeutic modality. *Front. Pediatr.* 3:58. doi: 10.3389/fped.2015.00058

Vogt, M., Puntschart, A., Geiser, J., Zuleger, C., Billeter, R., and Hoppeler, H. (2001). Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J. Appl. Physiol.* 91, 173–182. doi: 10.1152/jappl.2001.91.1.173

West, J. B. (2006). Human limits for hypoxia: the physiological challenge of climbing Mt. Everest. *Ann. N. Y. Acad. Sci.* 899, 15–27. doi: 10.1111/j.1749-6632.2000.tb06173.x

West, J. B. (2012). High-altitude medicine. *Am. J. Respir. Crit. Care Med.* 186, 1229–1237. doi: 10.1164/rccm.201207-1323CI

Wiesner, S., Haufe, S., Engeli, S., Mutschler, H., Haas, U., Luft, F. C., et al. (2010). Influences of normobaric hypoxia training on physical fitness and metabolic risk markers in overweight to obese subjects. *Obesity* 18, 116–120. doi: 10.1038/oby.2009.193

Yadav, V., Matsakas, A., Lorca, S., and Narkar, V. A. (2014). PGC1β activates an antiangiogenic program to repress neoangiogenesis in muscle ischemia. *Cell Rep.* 8, 783–797. doi: 10.1016/j.celrep.2014.06.040

Yoshioka, M., Tanaka, H., Shono, N., Snyder, E. E., Shindo, M., and St-Amand, J. (2003). Serial analysis of gene expression in the skeletal muscle of endurance athletes compared to sedentary men. *FASEB J.* 17, 1812–1819. doi: 10.1096/fj.02-1200com

Zoll, J., Ponsot, E., Dufour, S., Doutreleau, S., Ventura-Clapier, R., Vogt, M., et al. (2006). Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts. *J. Appl. Physiol.* 100, 1258–1266. doi: 10.1152/japplphysiol.00359.2005

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