Angiopoietins and Skeletal Muscle Function

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DEDICATION

To my mother Mina and to my sister Golroo, this thesis is the fruit of the sacrifices you made.

Thank you for having faith in me.

ABSTRACT

Angiopoietins are ligands for the endothelial cell-specific Tie-2 receptors. Angiopoietin-1 (Ang-1) activates Tie-2 receptors in the vasculature and promotes endothelial cell survival, proliferation, migration and differentiation. Angipoietin-2 (Ang-2) is synthesized mainly by endothelial cells and antagonizes Ang-1-induced Tie-2 receptor activation. In special circumstances, Ang-2 activates Tie-2 receptors and promotes angiogenesis. In this thesis, I address the regulation and functional significance of angiopoietins and Tie-2 receptors in normal and regenerating skeletal muscles. I describe first that skeletal muscle progenitor cells produce Ang-1 and Ang-2 and express Tie-2 receptors. Skeletal muscle Ang-1 and Ang-2 production increases significantly during progenitor cell differentiation to myotubes. Systemic inflammatory conditions such as severe sepsis trigger significant decline in skeletal muscle Ang-1 and Tie-2 levels while simultaneously inducing Ang-2 production through NFκB-dependent pathways. Skeletal muscle Ang-2 production is also upregulated by oxidative stress. In-vitro experiments using isolated skeletal muscle progenitors reveal that both Ang-1 and Ang-2 promote survival and differentiation of these cells but only Ang-1 induces proliferation and migration of muscle progenitors. These effects are mediated in part through phosphorylation of muscle-derived Tie-2 receptors and activation of the PI-3 kinase/AKT and ERK1/2 signaling pathways. In cardiotoxin-induced necrotic muscle injury model in mice, administration of adenoviruses expressing Ang-1 four days after the initiation of muscle injury elicits significant improvement of muscle regenerative capacity, increased angiogenesis and complete recovery of muscle contractility. These results uncover a novel and important role for Ang-1 in the promotion of skeletal muscle regeneration through enhancement of both, angiogenesis and myogenesis.

RESUMÉ

Les Angiopoétines sont des ligands pour les cellules endothéliales spécifiques aux récepteurs Tie-2. L'angiopoétine-1 (Ang-1) active les récepteurs Tie-2 dans la vasculature et favorise la survie, la prolifération, la migration et la différentiation. L'Angiopoétine-2 (Ang-2) est synthétisé principalement par les cellules endothéliales et antagonise l'activation des récepteurs Tie-2 induits par Ang-1. Dans des circonstances spéciales, Ang-2 active les récepteurs Tie-2 et favorise l'angiogénèse. Dans cette thèse, j'adresse la régulation et la signification fonctionnelle des Angiopoétines et des récepteurs Tie-2 dans des muscles squelettiques normaux et en régénération. Je décris en premier que les cellules souches musculaires squelettiques produisent Ang-1 et Ang-2 et expriment les récepteurs Tie-2. La production d'Ang-1 et Ang-2 du muscle squelettique augmente de façon significative pendant la différenciation des cellules souches en myotubes. Les conditions d'inflammation systémique telle que la septicémie sévère entraîne une baisse significative des niveaux d'Ang-1 et Tie-2 dans le muscle squelettique et induit simultanément une production d'Ang-2 à travers la voie de signalisation NFκB dépendante. La production d'Ang-2 des muscles squelettiques est aussi sur-régulée par le stress oxydatif. Les expériences in-vitro qui utilisent les ascendants isolés de muscles squelettiques révèlent que ensemble Ang-1 et Ang-2 favorisent la survie, la différentiation de ces cellules mais que seulement Ang-1 induit la prolifération et la migration des muscles ascendants. Ces effets sont négociés partiellement à travers la phosphorylation des récepteurs Tie-2 dérivés de muscles et l'activation des voies de signalisation PI-3 Kinase/AKT et ERK1/2. Dans le modèle cardiotoxique nécrotique induit de muscle blessé chez la souris, l'administration d'adénovirus exprimant Ang-1 quatre jours après l'initiation du muscle blessé montre une amélioration significative de la capacité régénérative du muscle, augmentant l'angiogenèse et la récupération complète de la contractilité du muscle. Ces résultats dévoilent un nouveau et important rôle d'Ang-1 dans la promotion de la régénération du muscle squelettique à travers l'augmentation de l'angiogenèse et de la myogenèse.

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AKT	Protein kinase B
Ang/ANGPT	Angiopoietin
AP-1	Transcription factor activator protein-1
BHLH	Basic helix-loop-helix domain containing
BMP	Bone morphogenetic protein
CARD	Caspase recruitment domain
COPD	Chronic obstructive pulmonary disease
CCND1	Cyclin D1
DMD	Duchenne muscular dystrophy
elF4E-BP1	Elongation factor 4E binding protein 1
eNOS	Endothelial nitric oxide
EFNA4	Ephrin-A4
ERK1/2	Extracellular signal-regulated kinase ½
FAK	Focal adhesion kinase
FEV1	Forced expiratory volume in one second
FGF	Fibroblast growth factor
FVC	Forced vital capacity
GADD	Growth arrest and DNA-damage-inducible factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GBP	Guanylate binding protein
GRB	Growth factor receptor-bound protein
GSK3β	Glycogen synthase kinase 3 beta
HGF	Hepatocyte growth factor
HNE	4-hydroxy-2-nonenal
HSM	Human skeletal myoblasts
HSPGs	Heparin sulphate proteoglycans
HUVEC	Human umbilical vein endothelial cell
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide
KLF2	Kruppel-like factor 2
KITLG	KIT ligand
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAFbx	Muscle atrophy F-box
MDA	Malonaldehyde
MHC	Myosin heavy chain
Mn-SOD	Manganese superoxide dismutase
mTOR	Mammalian target of rapamycin
MuRF	Muscle ring finger
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa beta

NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PI	Phosphatidylinositol/phosphoinositide
PI3-Kinase	Phosphatidylinositol-3' kinase
PlGF	Placental growth factor
PPAP	Phosphatidic acid phosphatase
PtdIns	Phosphatidylinositol
PTEN	Phosphoinositide-lipid 3-phosphatase
Rho	Ras-homologous
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S6K	p70 S6 kinase
SAPK/JNK	c-Jun N-terminal kinases/stress-activated kinases
SH	Src homology
SHIP	SH2-containing inositol polyphosphate 5-phosphatase
SE	Standard error
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
TA	Tibialis anterior
TGF-β	Transforming growth factor-beta
THBS	Thrombospondin
Tie	Tyrosine kinase with immunoglobulin and EGF-like domains
TNFα	Tumour necrosis factor alpha
TNFSF	TNF super family
Tyr	Tyrosine
UPS	Ubiquitin-proteosomal system
VEGF	Vascular endothelial growth factor
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VL	Vastus lateralis

CONTRIBUTIONS OF AUTHORS

This thesis was prepared according to principles outlined in McGill University's "Guidelines for Thesis Preparation." The format conforms to "manuscript-based thesis" option. The contributions of each author within a chapter are outlined below. Authors are designated by their initials.

Chapter 1 – Introduction and Literature Review

MM wrote the introduction and literature review. SNH provided comments and provided editorial assistance.

Chapter 2 – Regulation of Angiopoietin Expression by Bacterial Lipopolysaccharide

This research was originally published in the American Journal of Physiology - Lung Cellular and Molecular Physiology and is used in accordance with the copyright policy outlined by the American Physiological Society. It can be located under the citation: Mofarrahi M, Nouh T, Qureshi S, Guillot L, Mayaki D, Hussain SN. Regulation of angiopoietin expression by bacterial lipopolysaccharide. Am J Physiol Lung Cell Mol Physiol. 294(5):L955-L963, 2008. Human muscle cell experiments and protein analyses were performed by MM. Animal experiments were performed by MM and TN. RNA analyses were performed by MM and DM. Epithelial cell culture experiments were performed by SQ and LG. SNH supervised all research. The manuscript was written by MM and SNH.

Chapter 3 – Angiogenic factors in limb muscles of chronic obstructive pulmonary disease patients: Roles of angiopoietin-2

A portion of this research has been accepted in July 2011 for publication in the journal Plos One.
All skeletal muscle cell culture experiments and protein analyses were performed by MM. RNA expression experiments were analyzed by MM and DM. Microarray experiments were performed by Montreal Genome Centre and the results were analyzed by MM and SNH. FM provided and organized the delivery to our labs of human muscle biopsies from COPD patients and control subjects. The manuscript was written by MM and SNH.

Chapter 4 –Angiopoietin-1 promotes muscle regeneration and myoblast differentiation through complementary pathways involving Tie2

This research has recently been completed and is being prepared for submission to the journal Nature Medicine. All animal experiments were performed by MM. Muscle contractility measurements were performed by MM and GD. Immunohistochemistry experiments were performed by MM and AEP and experiments were supervised by ED. Quantification of morphological indices of

muscle injury and regeneration were performed by MM. In-vitro experiments shown in Figures 3, 17, 18, 19 and 20 were designed by MM and SNH and conducted by JMM and CDK at Duke University. Microarray experiments were performed by Montreal Genome Centre and the results were analyzed by MM and SNH. The manuscript was written by MM and edited by JMM and SNH.

Chapter 5 – Original Contributions to Scientific Knowledge and Discussion

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AEP-Amy E. Pickett
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SNH – Sabah N. Hussain
SQ – Salman Qureshi
TN – Thamir Nouh

OTHER CONTRIBUTIONS

In addition to the original contributions to scientific knowledge contained in this thesis, I have participated in several other published studies. These studies were completed in the laboratories of Dr. S. Hussain, under his supervision and in collaboration with several other faculty members, research fellows, and graduate students. They are as follows:

1. Debigare R, F. Maltais, C.H. Cote, A. Michaud, M.A. Caron, **M. Mofarrahi**, P. Leblanc and S.N.A. Hussain. Profiling of mRNA expression in quadriceps of patients with COPD and muscle wasting. COPD 5(2):75-84, 2008

2. Kim HC, **M. Mofarrahi**, S.N.A. Hussain. Skeletal muscle dysfunction in patients with chronic obstructive pulmonary disease. Int J. Chron. Obstruct. Pulmon.Dis. 3(4):637-658, 2008

3. Abdel-Malak NA, **M. Mofarrahi**, D. Mayaki, L.M. Kachigian and S.N.A. Hussain. Early growth factor-1 regulates angiopoietin-1-induced endothelial cell proliferation, migration and differentiation. Arterioscler.Thromb.Vasc. Biol. 29(2):209-216, 2009.

4. Hussain S.N.A., **M. Mofarrahi**, I. Sigala, H.C. Kim, T. Vasssilakopoulos, F. Maltais, I. Bellenis, R. Chaturvedi, S.B. Gottfried, P. Metrakois, G. Danialou, S. Matecki, S. Jaber, B.J. Petrof, P. Goldberg. Mechanical ventilation-induced diaphragm disuse in humans triggers autophagy. Am. J. Resp. Crit. Care Medicine 182(11):1377-86, 2010

5. Mofarrahi M*, Kim HC*, , Vassilakopoulos T, Maltais M, Sigala I, Debigare R, Bellenis I, Hussain SN. Expression and functional significance of nicotinamide N-

methyl transferase in skeletal muscles of patients with chronic obstructive pulmonary disease. Am. J. Resp. Crit. Care Medicine 181(8):797-805, 2010. *Co-first authors.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The Angiopoietin/Tie System

1.1.1 Structure of angiopoietins and Tie receptors

The angiopoietin-Tie system is comprised of four secreted ligands, including angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), angiopoietin-3 (Ang-3), and angiopoietin-4 (Ang-4), and two type I tyrosine kinase receptors known as Tie-1 and Tie-2. Tie-2 receptors are expressed at very high levels on the surface of vascular endothelial cells. All angiopoietins are able to bind to it. Tie-1 is an orphan receptor that has been shown to interact with and regulate the activity of Tie-2 receptors (131, 291, 312). Tie-2 is a receptor tyrosine kinase (RTK) containing Ig-like loops, EGF-like motifs, and fibronectin type-like repeats. Tie receptors are abundantly expressed in all endothelial cells, although weak expression has been detected in neutrophils, sensory neurons, and ganglia (84, 155, 193, 194, 203, 297, 298).

Angiopoietins are soluble secreted proteins that can be found as dimers or higher order multimers. Ang-1 (ANGPT1) is mainly produced by vascular smooth muscle cells, pericytes, fibroblasts, thyrocytes and adipocytes, while Ang-2 (ANGPT2) is primarily produced by endothelial cells (70, 89, 194, 218, 277, 372). Upon secretion, Ang-1 is incorporated into the extracellular matrix via a linker peptide region (379). Under reduced conditions, Ang-1 can be detected as a 70kD glycoprotein (183). Ang-1 is made up of a carboxyl fibrinogen-like domain responsible for binding Tie-2 receptors, a central coiled domain that is required for Ang-1 dimerization, and, finally, a short amino terminal that is involved in Ang-1 secretion and in the formation of Ang-1 multimers by superclustering. Since Ang-1 dimers and trimers have been shown to be inactive, oligomerization of Ang-1, so that

it contains at least four monomers, is critical for its binding and activation of Tie-2 receptors (183, 270). In addition to full-length Ang-1 monomers of 1.5 kilobases and 498 amino acid residues, thus far three smaller splice variants of 1.3, 0.9, and 0.7 kilobases and 367, 285 and 154 amino acid residues respectively, have been identified. Their functions have yet to be determined. The 1.3 and 0.9 kilobase monomers lack the full form of fibrinogen-like and coiled-coiled domains, respectively, and it is suggested that they act as dominant-negative regulators of Ang-1 (147).

Ang-2 is structurally similar to Ang-1 and consists of 496 amino acids. An alternative splice variant of Ang-2 contains 443 amino acids and lacks the coiled-coiled domain. It has been shown to inhibit Ang-1 induced Tie-2 activation (179). Ang-2 exists predominantly as a dimer that is stored in Weibel-Palade bodies in the cytoplasm of endothelial cells and, upon stimulation, is released (98). Both Ang-1 and Ang-2 are able to bind Tie-2 receptors, on the same domain with similar affinities, but Ang-2 induces weak phosphorylation of the receptor and blocks Ang-1 induced Tie-2 phosphorylation in a competitive manner (70, 96, 218). In the vascular system, it has been suggested that Ang-2 functions mainly as a modulator of responses elicited by other stimuli, such as thrombin, tumour necrosis factor alpha (TNFα), and vascular endothelial growth factor (VEGF) (97, 146). In addition, it has also been proposed that Ang-2 acts as a natural Ang-1 antagonist and reverses Ang-1-induced endothelial cell quiescence, thereby rendering these cells more susceptible to certain stimuli (218, 263, 302). However, there is also evidence that Ang-2 by itself can elicit primary responses, such as increased apoptosis and enhanced vascular leakage, without the presence of additional stimuli (66, 134, 178).

The nature of interactions between Ang-2 and Tie-2 receptors is not fully understood. For instance, *in vitro* assays have revealed that when Tie-2 receptors are ectopically expressed in non-endothelial cells (NIH 3T3 fibroblasts), they are capable of responding to Ang-2 concentrations that are much lower than those capable of inducing receptor phosphorylation in endothelial cells (178, 218). Moreover, in fibrin clots, prolonged exposure to Ang-2 induces Tie-2 phosphorylation and when endothelial cells are grown in collagen gels, Ang-2 stimulates both chemotaxis and capillary-like tube formation, both of which are considered pro-angiogenic stimuli (236, 347). These experiments suggest a dual role for Ang-2, which depends on the experimental context and the cell type in use.

Murine Ang-3 and the human ortholog Ang-4 are other members of the angiopoietin family (359). Ang-3 exhibits wide tissue distribution and is mainly produced by peri-endothelial cells. Ang-3 is tethered to the cell surface through interaction of its coiled-coiled domain with heparan sulfate proteoglycans, perlecan in particular. Since the soluble form of Ang-3 does not elicit much bioreactivity, its interaction with the cell membrane seems to be crucial. Ang-3 is also a ligand of Tie-2 receptors and activates Tie-2 in non-endothelial cells. In endothelial cells, competitive binding of Ang-3 to Tie-2 receptors has been shown to block Ang-1-induced Tie-2 activation, thereby exerting an antagonistic effect on Ang-1 signalling (377, 378). Ang-4 is mainly expressed in human lungs and its cellular source has yet to be identified. Ang-4 is able to bind and induce Tie-2 activation in endothelial cells, promoting survival and migration (202).

1.1.2 Biological roles of angiopoietins and Tie receptors

1.1.2.1 Embryonic development: Targeted disruption of Tie-2 or Ang-1 genes is associated with extensive haemorrhage, detachment of the endothelium, loss of endothelial cell integrity, and failure to recruit pericytes, thereby causing embryonic lethality at days E11-12 (164, 299, 303, 336). Exact roles of Ang-2 in embryonic vascular development are as yet unclear. The observation that transgenic mice overexpressing Ang-2 develop a phenotype that is similar to that seen in Tie-$2^{-/-}$ and Ang-$1^{-/-}$ mice suggests that Ang-2 plays a role as an Ang-1 antagonist during vascular development (218). However, subsequent research has revealed that genetic deletion of Ang-2 triggers major lymphatic vascular defects, the development of post-natal chylous ascites, and subsequent death (107). Taken together, these observations suggest that Ang-2 plays a critical role in lymphatic vascular development. Observations that Ang-1 overexpression is able to rescue lymphatic, but not angiogenic, defects of Ang-$2^{-/-}$ mice, suggest that both angiopoietins might function as agonists for Tie-2 receptors in lymphangiogenesis.

1.1.2.2 *In vitro* angiogenesis: Angiogenesis is the culmination of several processes that are designed to elicit degradation of basement membranes, proliferation, migration, adhesion, and remodelling of endothelial cells, eventually resulting in neovascularization. Using *in vitro* reductionist models, numerous investigators have studied how angiopoietins alter angiogenesis-related processes. There is no doubt that Ang-1 inhibits apoptosis triggered by stimuli such as serum deprivation, TNFα, and oxidized low density lipoprotein (LDL) (132, 133, 177, 262). Ang-1 also functions as a chemoattractant to promote endothelial cell migration, as measured by both the classical Boyden chamber assay and the wound-healing assay (49, 53, 104, 372).

Moreover, Ang-1 induces endothelial cell sprouting and differentiation into tube-like structures in 2D and 3D matrices (15, 53, 176, 188). There are no clear conclusions regarding the influence of Ang-1 on endothelial cell proliferation. In murine brain capillary endothelial cells, Ang-1 elicits strong proliferative responses, whereas in adrenal-cortex-derived microvascular endothelial and human umbilical vein endothelial cells, responses are milder (168, 188). There are also studies that report no improvement in the proliferative capacity of endothelial cells in the presence of Ang-1 (104, 372).

While the influence of Ang-1 on endothelial cell proliferation is debatable, there is little discussion as to the effectiveness of Ang-1 in regulating endothelial cell vascular leakage. There is evidence that in endothelial cells Ang-1 triggers mobilization of PECAM-1 to junction areas, inhibition of VE-cadherin phosphorylation, and tightening of endothelial cell junctions, resulting in attenuation of VEGF- and thrombin-induced endothelial cell permeability (108). Improved vascular integrity and inhibition of vascular leakage by Ang-1 has been confirmed in transgenic mice that express Ang-1 in the skin and Ang-1 has recently been linked to significant decreases in both the size and number of endothelial cell gaps(16, 352).

1.1.2.3 *In vivo* angiogenesis: Investigators have used recombinant proteins, adenoviruses, naked plasmids, and transgenic animals to assess how Ang-1 modulates *in vivo* angiogenesis. In developing mice, localized Ang-1 overexpression in the liver elicits significant enlargement and sprouting in the hepatic arterial circulation and induces portal vein dilation (367). In adult mice, Ang-1 stimulates *in viv*o vascular remodelling, vascular enlargement, enhanced wound healing, and increased lymphangiogenesis (50, 56, 57, 321, 337, 353). Moreover, Cho et al. (57) have

reported that an engineered version of Ang-1 (COMP-Ang-1) in a diabetes mouse model enhances wound healing through increased angiogenesis and lymphangiogenesis. The same group also found that COMP-Ang-1 protein significantly enhances angiogenesis in a corneal micropocket assay (55).

1.1.2.4 Pathologic angiogenesis: Ang-1 expression is enhanced in rheumatoid arthritis (RA), where Ang-1 protein is found in the synovial lining layer and in cells within the sub-lining synovial tissue (119). In addition, many cancer cell lines and tumour tissues, such as breast cancer cells and tissues (147), small cell lung carcinoma (342), cervical cancer (319), and prostate cancer (42), produce Ang-1. Inhibition of Ang-1 expression by anti-sense RNA or by overexpression of the extracellular domain of Tie-2 results in attenuation of tumour growth in many xenograph models (210, 211, 320).

1.1.2.5 Inflammation: There is increasing interest in studying the influence of Ang-1 on innate immune responses. Within minutes of Ang-1 exposure, endothelial cell P-selectin expression increases slightly, resulting in small increases in leukocyte adhesion (203). However, when Ang-1 is co-incubated for hours with VEGF or TNFα, leukocyte adhesion to endothelial cells, migration of leukocytes across endothelial cells, and expressions of adhesion molecules and pro-inflammatory mediators (ICAM-1, VCAM-1, E-selectin and tissue factor) are all strongly inhibited (108, 181, 182). This anti-inflammatory effect of Ang-1 is also seen in transgenic mice overexpressing Ang-1, as well as in mice injected with Ang-1 adenoviruses (167, 353, 373).

In a recent paper McCarter *et al*. (228) have demonstrated that a cell-based gene therapy that injects cells overexpressing Ang-1 into airways of rats with acute lung injury results in major improvements in morphological, biochemical, and molecular indices of lung injury and inflammation. They also found that degrees of lung injury and mortality are much higher in heterozygous Tie-2-deficient mice as compared to wild type mice, further confirming the importance of the Ang-1/Tie-2 receptor system in attenuating inflammation. Mechanisms through which this anti-inflammatory effect is mediated remain unknown. There is, however, a suggestion that Ang-1 may trigger selective inhibition of nuclear factor-kappa beta (NFκB) activation through Tie-2 receptors and that this effect is meditated by direct protein-protein interactions between Tie-2 receptors, A20 binding inhibitor of NFκB activation-2 (ABIN-2), and NFκB protein subunits (150, 341).

While Ang-1 has been shown to have some anti-inflammatory qualities, Ang-2 is a key regulator of vascular inflammation. The presence of Ang-2 enables cultured endothelial cells to express ICAM1 and VCAM1, which, in turn, induces monocyte adhesion in response to TNFα (97). Mice deficient in Ang-2 elicit a significantly attenuated inflammatory response to thioglycollate-induced or *Staphylococcus aureus*-induced peritonitis; injecting them with Ang-2 restores inflammatory responses (97). Pro-inflammatory effects of Ang-2 are further illustrated by the observation that injection of Ang-2 protein *in vivo* elicits significant increases in oedema formation in the mouse paw (288).

1.1.3 Tie-2 Signalling

Upon ligation of ligands, Tie-2 receptors dimerize and cross-phosphorylate multiple tyrosine residues in the cytoplasmic domain. Phosphorylated tyrosine residues, in turn, create docking sites for several cytoplasmic adaptor proteins. In murine endothelial cells, phosphorylation of Tyr^{1100} enables Tie-2 receptors to recruit through their SH2 domains growth factor receptor-bound proteins (GRB) 2 and 7, as well as the p85 subunit of PI3-kinase, while Grb14 binds to phosphorylated Tyr^{814} and Tyr^{1106} residues of Tie-2 receptors (163). Dok-R protein has been shown to bind phosphorylated Tyr^{1106} through its PTB domain. The presence of a pleckstrin homology (PH) domain in Dok-R enables it to localize to the plasma membrane and, as a result, further enhance its binding to Tie-2 (161, 162). The activity of Tie-2 receptors is also regulated by SH2-containing tyrosine phosphatase-2 (SHP-2), which can associate with Tie-2 through activated Tyr^{814} and Tyr^{1111} residues (163). Once SH2 domains of SHP-2 interact with Tie-2 receptors, SHP-2 phosphatase activity increases significantly (265). When the association of SHP-2 and Tie-2 is disrupted by mutation or truncation of SHP-2 binding sites, Tie-2 receptors undergo increased autophosphorylation, resulting in enhanced activation of downstream signalling pathways (253).

Vascular endothelial protein tyrosine phosphatase (VE-PTP), which is an endothelial cell-specific phosphatase that directly interacts with and regulates the activity of Tie-2, is another phosphatase that interacts with Tie-2 receptors (28, 91). Moreover, in human endothelial cells, Tie-2 activation leads to recruitment of Grb2 and SHP-2 by Tyr^{1101} and Tyr^{112} residues, respectively. These adaptor proteins, in turn, link Tie-2 receptors to AKT and mitogen-activated protein kinase (MAPK) signalling pathways (145, 191, 293). The p85 regulatory subunit of type I PI3-kinase

binds to Tyr^{1101} of Tie-2 receptors and, to a lesser extent, to Tyr^{1112}, resulting in the activation of PI3-kinase activity and its downstream signalling pathways, including activation of AKT (191). Ang-1-induced activation of Tie-2 receptors also triggers phosphorylation and recruitment of the adaptor protein ShcA to Tie-2. This association involves Tyr^{1101} and results in the promotion of endothelial cell sprouting and migration (15).

Exposure to Ang-1 protein has been shown to trigger activation of several pathways, the majority of which are mediated through Tie-2 receptors. However, selective pathways are also activated as a result of ligation of various integrins by Ang-1. The following pathways have been shown to be activated in endothelial cells exposed to recombinant Ang-1 protein:

1.1.3.1 The phosphoinositide 3-kinase pathway: PI3-kinases are a ubiquitous family of lipid kinases that generate 3'-phosphorylated phosphoinositides (PIs) by catalyzing the addition of a phosphate group to the 3-hydroxyl position of the inositol ring of membrane-localized phosphatidylinositol (PtdIns) (276, 296). Nine mammalian PI3-kinases have been identified to date and are categorized into three main classes (I, II, and III), based on sequence homologies, regulation, and lipid specificities (82, 276). PI3-kinases are heterodimeric proteins that contain a regulatory subunit and a catalytic subunit (12, 82). Four functionally different lipid products, namely PtdINs-3-P, PtdIns-3,4-P_2, PtdIns-3,5-P_2, and PtdIns-3,4,5-P_3, act as docking sites for different signalling molecules that contain a PH domain and are involved in an array of cellular functions including inflammation, migration, proliferation, survival, and glucose metabolism (43, 106, 174, 276). In response to growth factors, class I enzymes generate PtdIns-3,4,5-P_3 by phosphorylating PtdIns-4,5-P_2 (139, 296). These

enzymes are composed of a p85 regulatory subunit and a p110 subunit that contains a lipid-directed catalytic domain. The p85 subunit interacts with receptor phosphotyrosines through its SH2 domain and recruits the catalytic subunit to activate tyrosine kinase receptors (82). Termination of class I PI3-kinase activity through PtdIns-3,4,5-P3 dephosphorylation is mediated through two inositol phosphatases, SH2-containing inositol polyphosphate 5-phosphatase (SHIP) and phosphoinositide-lipid 3-phosphatase (PTEN) (287, 330).

Class I enzymes can also activate the mammalian target of rapamycin (mTOR)/p70S6 kinase (S6K) signalling pathway, which is involved in the regulation of cell growth, insulin metabolism, and protein synthesis (9, 272, 295, 376). Several reports, including one from the laboratory in which this thesis was completed (Hussain laboratory), have confirmed that in endothelial cells the class I PI3-kinase pathway is activated downstream from Tie-2 receptors in response to Ang-1 and Ang-2, and that this pathway promotes Ang-1-triggered endothelial cell migration, adhesion, differentiation, and survival(104, 177, 262).

1.1.3.2 The mitogen-activated protein kinase pathway: Mitogen-activated protein kinases are a family of serine/threonine kinases. Extracellular signal-regulated kinase 1/2 (ERK1/2,) c-Jun N-terminal kinases/stress-activated kinases (SAPK/JNK) and p38 MAPK are among members of this family that have been extensively studied. ERK 1 (p44) and ERK 2 (p42) are ubiquitously expressed proteins that are involved in cellular growth and transformation, differentiation, survival, and cytoskeletal reorganization. Spatiotemporal regulation of this pathway determines the outcome of its activation. For example, in neuronal cells, while transient activation of this pathway induces cell proliferation, its sustained activity triggers differentiation or

apoptosis (224, 267, 286, 333). So far fifty substrates have been linked to the ERK1/2 signalling pathway, which suggests that it plays diverse biological roles. A large number of these are transcription factors that are involved in cell proliferation (209).

The SAPK/JNK and p38 MAPK pathways are mainly activated by stress signals such as osmotic pressure, heat, reactive oxygen and nitrogen species (ROS and RNS), ultraviolet radiation, and inflammatory cytokines. Unlike the situation with the ERK1/2 pathway, growth factors elicit weak responses in these pathways (69, 199, 317, 354). SAPK/JNK kinases are mainly activated downstream of Rac1/2 and Cdc-42, members of the Ras-homologous (Rho) family, which are strong promoters of cell migration (17, 325, 358). The SAPK/JNK family is compromised of up to ten isoforms, a result of alternative splicing of JNK1, 2 and 3 genes (69, 354). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is mainly present in the heart, brain, and testes (69). The p38 MAPK family includes p38α, β, γ (SAPK3/ERK6), and δ (SAPK4). Except for the γ isoform, which is exclusively expressed in skeletal muscle, other members of this family are ubiquitously expressed (126, 153, 225).

The Hussain laboratory was first to conclude that the MAPK pathway is an important target of activation by the Ang-1/Tie-2 receptor system. Harfouche *et al.* (132) demonstrated that exposure of human umbilical vein endothelial cells (HUVECs) to Ang-1 triggers transient (within 10 to 15 minutes) increases in phosphorylation of the ERK1/2 pathway (15-fold) and p38 MAPK (5-fold) pathways and that inhibition of the PI3-kinase pathway attenuates Ang-1-induced ERK1/2 phosphorylation at a level upstream from Raf1 and MEK1/2 kinases. These authors also demonstrated that inhibition of the ERK1/2 pathway eliminates inhibitory effects

of Ang-1 on the activities of caspase 3, 7 and 9, indicating that the ERK1/2 pathway exerts anti-apoptotic effects in response to Ang-1 exposure. In comparison, inhibition of the p38 MAPK pathway augments anti-apoptotic effects of Ang-1 in HUVECs, indicating that this pathway serves as a pro-apoptotic stimulator in this setting (132). In a subsequent study, Harfouche *et al.* (135) concluded that the SAPK/JNK pathway is also activated in response to Ang-1 exposure in HUVECs, however, whether or not it regulates Ang-1-induced endothelial cell survival was not evaluated in that study. Activation of the SAPK/JNK pathway in response to Ang-1 exposure in endothelial cells was later confirmed (3).

More recent studies from the Hussain laboratory (1-3) have revealed that important crosstalk occurs between the p38 and the ERK1/2 pathways in endothelial cells exposed to Ang-1. In this crosstalk, p38 MAPK exerts a negative influence on ERK1/2 activation. It was also concluded that the biological effects of Ang-1 that were observed in the presence of p38 MAPK pathway inhibition might be due to augmented ERK1/2 activation, a result of removal of inhibitory effects of the p38 MAPK pathway on the ERK1/2 pathway. Exact mechanisms responsible for this negative crosstalk remain to be investigated.

1.1.3.3 Mammalian target of rapamycin pathway: Mammalian target of rapamycin is a Ser/Thr kinase that regulates cell growth and metabolism. mTOR exists as two complexes: the rapamycin-sensitive mTORC1, which contains mTOR, raptor and mLST8 proteins; and the rapamycin-insensitive mTORC2 complex, which contains mTOR, rictor, and mLST8 proteins (376). The mTOR network is inhibited by two proteins - hamatrin (TSC1) and tuberin (TSC2) - while three upstream pathways (PI-3-kinase, Erk1/2, and amino acid sensing) activate mTOR signalling (52, 92). The PI-

3 kinase/AKT pathway inactivates the TSC2 complex, resulting in mTOR phosphorylation at Ser^{2448} (222, 313). Activation of the ERK1/2 pathway directly phosphorylates TSC2, thereby inactivating the TSC1-TSC2 complex (216). mTOR also appears to interact with the amino acid sensing pathway to activate downstream effectors.

The best-characterized targets of the mTOR network are the translation regulators, including p70S6 kinase 1 (S6K1) and elongation factor 4E binding protein 1 (elF4E-BP1). Phosphorylation of 4E-BP1 releases inhibitory effects on elF4E, which then triggers protein translation. Activated S6K1 phosphorylates the ribosomal protein S6, resulting in increased translation. The active mTOR network also regulates ribosome biogenesis, glucose and fat metabolism, autophagy, transcription, and actin organization (376). Moreover, several recent reports have confirmed that mTOR network inhibition by rapamycin attenuates VEGF-induced EC proliferation and sprouting (79, 122).

Despite the importance of the mTOR network in angiogenesis, specific roles of this network in Ang-1/Tie-2 signalling are not yet clear. The Hussain laboratory has recently reported that Ang-1 exposure in HUVECs elicits significant increase in mTOR phosphorylation on Ser^{2481} and P70S6 kinase phosphorylation on Thr^{389} (1). In addition, Ang-1-induced P70S6 kinase phosphorylation is significantly attenuated in the presence of wortmannin (PI-3 kinase inhibitor) and rapamycin (mTORC1 inhibitor) (1), suggesting that P70S6 kinase is activated by both mTORC1 and the PI-3 kinase pathway. The Hussain laboratory has also concluded that the mTORC1 network may participate in Ang-1-induced alterations in the regulation of several cellular networks that are activated in endothelial cells (1).

1.1.3.4 The DOK-R pathway: A novel docking molecule, Dok-R, has been shown to interact with the Tyr^{1107} residue of Tie-2 receptors and that this interaction leads to recruitment of the Nck adaptor protein, RasGAP, and p21 activated protein kinase (PAK-1), leading to increased EC migration (162, 226). The Hussain laboratory has confirmed that Ang-1 exposure in HUVECs triggers significant increases in PAK-1 phosphorylation on Ser^{199}, Ser^{144}, and $Thre^{423}$ and that this phosphorylation is associated with induction of endothelial cell migration, further confirming involvement of the DOK-R/Nck/PAK-1 pathway in the biological response to Ang-1 (135).

1.1.3.5 Focal adhesion kinase (FAK): p125 focal adhesion kinase (FAK) is a member of non-receptor protein kinases that play a key role in regulating dynamic changes in actin cytoskeletal organization during cellular migration and adhesion (301). FAK is activated by growth factors that induce its tyrosine phosphorylation (301). FAK activation triggers phosphorylation of paxillin, a cytoskeletal protein involved in actin-membrane attachment at sites of cell migration. Kim *et al.* (176) have reported that Ang-1 triggers significant increases in FAK and paxillin phosphorylation in endothelial cells in time- and dose-dependent fashions and that these rises in phosphorylation are mediated through the PI3-kinase pathway.

1.1.3.6 Soluble mediators: Very little is known about how the Ang-1/Tie-2 receptor system triggers release of soluble mediators from endothelial cells. Iivanainen *et al*. (154) were first to report that Ang-1 exposure elicits enhanced release of heparin binding EGF-like growth factor from endothelial cells and that this growth factor is involved in recruitment of smooth muscle cell migration towards endothelial cells during the course of angiogenesis. In a subsequent study, Kobayshi *et al.* (187)

described upregulation of hepatocyte growth factor (HGF) expression and its release from endothelial cells stimulated with Ang-1. They also implicated HGF in Ang-1-induced smooth muscle recruitment to newly-formed blood vessels. Abdel-Malak *et al.* (3) have recently described transient (peak in 1 hour) and significant upregulation of interleukin-8 (IL8) expression and release from endothelial cells stimulated with Ang-1. They also demonstrated that enhanced IL8 production is mediated through PI-3 and MAP kinases and that IL8 promotes endothelial cell migration through autocrine and paracrine mechanisms (3).

1.1.3.7 Transcription factors: Little is known regarding the nature of various transcription factors activated by the Ang-1/Tie-2 pathway. One report has documented signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5) activation by Ang-1 (192). STATs are continuously shuttled from the cytosol to the nucleus and, upon activation, are retained at the nucleus where they elicit changes in gene expression.

The Hussain laboratory has reported that Ang-1 induces Elk1 phosphorylation downstream from Erk1/2 (132). Elk-1 is a member of ETS-oncogene family that forms a ternary complex by binding serum response factors and serum response elements in various promoters. Abdel-Malak *et al*. (3) described activation of the transcription factor activator protein-1 (AP-1) in endothelial cells exposed to Ang-1, including c-Jun-c-Jun dimerization of AP-1 subunits. They also linked the PI-3 kinase, ERK1/2, and SAPK/JNK pathways to the activation of AP-1 and associated this transcription factor with induction of IL8 release (3). Sako *et al*. (292) described activation of promoter of Kruppel-like factor 2 (KLF2) in endothelial cells exposed to Ang-1 and linked its expression to the PI3-kinase pathway. Moreover, they also

found that deletion of KLF2 eliminates inhibitory effects of Ang-1 on VEGF-induced adhesion molecule expression and monocyte adhesion, suggesting that KLF2 mediates anti-inflammatory effects of Ang-1.

1.1.3.8 Roles of NADPH oxidase: Reactive oxygen species were originally considered toxic by-products of cellular metabolism. However, recent studies have indicated that ROS are produced transiently in response to receptor activation in a variety of cells and play important roles as second messengers downstream of RTKs (349). While ROS are likely to originate from several intracellular sources, including the mitochondrial electron transport chain, nitric oxide synthase (NOS), xanthine oxidase, and lipooxygenases, recent studies implicate an NADPH oxidase enzyme complex in the generation of ROS in response to RTKs in vascular cells (94). NADPH oxidase is an O_2^--generating enzyme complex that was initially characterized as the source of phagocytic respiratory bursts. In phagocytes, this enzyme consists of several subunits, including $g91^{phox}$, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac-1/2 (72). These subunits have also been identified in several non-phagocytic cells, including endothelial cells (151).

The Hussain laboratory was first to report that acute exposure of endothelial cells to Ang-1 elicits significant release of ROS and that NADPH oxidase is the main, but not sole, contributor to this ROS production (135). They also revealed that NADPH oxidase-derived ROS play important roles in regulating downstream signalling pathways, including AKT and MAPKs, and that these roles result in the promotion of Ang-1-induced endothelial cell migration (135). Two subsequent studies confirmed this observation and described an important role for NAPDH oxidase-derived ROS in Ang-1-induced angiogenesis and endothelial cell

differentiation (54, 184). Whether or not other ROS generating enzymes, such as xanthine oxidase, lipoxygenases, and mitochondrial-based oxidoreductases, are also involved in Ang-1 signalling in endothelial cells remains to be investigated.

1.1.3.9 Other signalling pathways: To explore the various cellular networks that may be regulated by the Ang-1/Tie-2 receptor system, Abdel-Malak *et al.* (1) exposed HUVECs to Ang-1 for four hours and then profiled the mRNA transcriptome using oligonucleotide microarrays. They described significant upregulation of eighty-six genes with STC1 (a protein involved in the regulation of intracellular Ca^{++} levels), BCL2A1 (an anti-apoptotic member of the BCL2 family), and CARD8 (of the caspase recruitment domain family) being the most highly induced (1). Other significantly upregulated Ang-1 genes included several proteins that are involved in cell cycle regulation, migration, survival, cell-to-cell interaction, and endothelial cell differentiation, such as VEGFC, VEGF receptor 1 (FLT1), platelet-derived growth factor A (PDGFA), PLAUR, ANGPTL4, IL8, CXCR4, Cyclin D1 (CCND1), KIT ligand (KITLG), ephrin-A4 (EFNA4), phosphatidic acid phosphatase type 2B (PPAP2B), and JAG1 (ligand for notch 1 receptors). Six transcription factors were also induced by Ang-1, including early growth response 1 (EGR1), Kruppel-like factor 2 (KLF2), v-jun sarcoma virus 17 oncogene homolog (avian) (JUN), ETS variant gene 5 (ETV5), basic helix-loop-helix domain containing, class B, 2 (BHLHB2), and transcription factor 8 (TCF8). Exposure to Ang-1 resulted in downregulation of forty-nine genes, including chromosome 8 open reading frame 4 (C8orf4, also known as TC1), serum deprivation response (phosphatidylserine-binding protein) (SDPR), a promoter of growth arrest, and cytochrome P450, family

26, subfamily B, polypeptide 1 (CYP26B1), which is involved in cellular differentiation (1).

Many pro-inflammatory, pro-apoptotic, and anti-proliferative genes are also downregulated by Ang-1, including TNF (ligand) superfamily, member 10 (TNFSF10, TRAIL), bone morphogenetic protein 4 (BMP4), growth arrest and DNA-damage-inducible factor, beta (GADD45B), thrombospondin 1 (THBS1), guanylate binding protein 1 (GBP1), cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) (CDKN2C), sulfatase 1 (SULF1), TTK protein kinase (TTK), budding uninhibited by benzimidazoles 1 homolog (yeast) (BUB1), and kinetochore associated 2 (KNTC2). Three transcription factors (inhibitor of DNA binding dominant-negative helix-loop-helix protein 1, 2, and 3) were also significantly downregulated by Ang-1 in endothelial cells (1). These results indicate that exposure to Ang-1 triggers coordinated responses designed to inhibit expressions of pro-apoptotic and anti-proliferative genes and to upregulate pro-proliferative, pro-angiogenic, and anti-apoptotic pathways.

1.1.4 Regulation of angiopoietin expression

Regulation of angiopoietin expression has been widely studied in relation to a variety of stimuli and in different cell types and a detailed discussion of the findings of these studies is beyond the scope of this introduction. Accordingly, discussion is limited to factors that are relevant to the vascular and musculoskeletal systems. Growth factors exert significant influences on Ang-1 expression. For instance, in an early study Enholm *et al.* (89) reported that in fibroblasts Ang-1 expressions decline upon serum, PDGF, EGF, and transforming growth factor-beta (TGFβ) stimulation

(89). In vascular smooth muscle cells, Nishishita and Lin (252) described significant upregulation of vascular smooth muscle Ang-1 expression in response to PDGF stimulation, a response that is inhibited by TGFβ.

In addition to growth factors, Ang-1 production is regulated by oxygenation levels. For example, in response to hypoxia and reoxygenation, Ang-1 expression declines in fibroblasts (89) and is significantly induced in vascular smooth muscle (278). In hypoxia-exposed rats, the Hussain laboratory reported that Ang-1 expressions decline significantly in the lung, liver, cerebellum, and heart, while mRNA levels of Ang-2 and Ang-3 increase significantly in the lung, kidney, and diaphragm (4). *In vitro* hypoxia also elicits significant upregulation of Ang-2 expression in endothelial and other cell types (195, 220, 221). These results suggest that hypoxia may elicit differential changes in angiopoietin expression, where Ang-1 is downregulated and Ang-2 is upregulated.

Inflammatory mediators are another group of factors that influence cellular Ang-1 production. There is general agreement that in *in vitro* settings Ang-2 expression in vascular cells is upregulated by pro-inflammatory cytokines and mediators. Indeed, several reports using cultured endothelial cells have confirmed that TNFα, thrombin, and angiotensin II upregulate endothelial Ang-2 expression (180, 220). Similarly, Ang-2 expressions in macrophages are significantly induced by interferon γ and prostaglandin E2 (148). Observations that plasma Ang-2 levels are elevated in patients with severe sepsis and in children with septic shock suggest that, in multiple organs, systemic inflammatory responses are accompanied by induction of Ang-2 (114, 254). Hegen *et al.* (140) have characterized a 585-base pair segment

of human Ang-2 promoter and demonstrated that Ets-1 and Elf1 transcription factors are strong enhancers of endothelial Ang-2 promoter activity. They have also suggested that negative regulatory elements also exist in the promoter, which may result in downregulation of Ang-2 promoter activity.

There are only a few studies that have explored *in vivo* influences of inflammatory mediators on vascular and non-vascular angiopoietin expression. Karmpaliotis *et al.* (170) found abundant Ang-2 expression in bronchial epithelial cells and alveolar macrophages and demonstrated that *in vivo* administration of *E. coli* lipopolysaccharide (LPS) into the lung triggers a decline in bronchial epithelial cell Ang-2 expression and upregulation of macrophage Ang-2 expression. These results indicate that pulmonary Ang-2 expression in response to a single inflammatory stimulus (LPS) depends on the type of cell being examined. The influence of LPS administration on Ang-2 expression in other organs and cell types remains to be investigated.

Studies describing pro-inflammatory mediator influences on Ang-1 expression have revealed contradictory results. For instance, in synovial fibroblasts, Ang-1 expression is upregulated by TNFα, through inductions of NFκB transcription factors (309). In another study, Brown *et al.* (36) analyzed a 3.2 kilobase unit of human Ang-1 promoter and found no binding sites for transcription factors that are associated with inflammatory mediators such as NFκB, STATs, NFAT and Ce/EBP. Instead, they found several binding sites for Ets and AP-1 transcription factors. They also revealed that exposure to TNFα, IL1β, or LPS significantly upregulates Ang-1 promoter activity in NIH3T3 fibroblasts and that mutation of a high-affinity binding

site for the transcription factor ESE-1 attenuates this response. Thus, it would appear that, at least in cultured cells, pro-inflammatory mediators may trigger significant upregulation of Ang-1 expression. However, their results contradict those of Karmpaliotis *et al.* (170) who described significant attenuation of epithelial Ang-1 expression in a murine model of LPS-induced acute lung injury.

Reasons behind these contradictory results are unclear but investigators have suggested that there may be organ-specific transcription factors that play a role in determining angiopoietin expression in a given organ. Contradictory findings also illustrate the need for a systematic study aimed at evaluating pro-inflammatory cytokine influences on organ-specific Ang-1 and Ang-2 production *in vivo*, so as to understand the overall contribution of differential angiopoietin expression to the pathogenesis of conditions such as septic shock, where pro-inflammatory cytokines play a central role in mediating tissue injury and organ dysfunction, eventually leading to high mortality. The fact that no information is as yet available regarding organ-specific differences in Ang-3 and Ang-4 expression further emphasizes the need to evaluate not only differences in Ang-1 and Ang-2 expression in response to pro-inflammatory cytokines, but also those of Ang-3 and Ang-4. In chapter 2 of this thesis, I address organ-specific effects of systemic administration of *E. coli* LPS and investigate the role of NFκB in LPS-induced septic shock on Ang-1, Ang-2, and Ang-3 (murine ortholog of Ang-4) expressions in mice. I chose to focus on angiopoietin responses to LPS because of the biological importance of LPS in the pathogenesis of septic shock, which is a major cause of mortality in intensive care units and accounts for more than 75,000 deaths per year in Canada.

1.1.4.1 Angiopoietins and skeletal muscles: Very little is known about angiopoietin expression and functionality in skeletal muscles. While it has been well established that angiopoietins are abundantly expressed in vascular cells, investigators have only recently focused on the regulation of angiopoietin expression in skeletal muscles. Lloyd *et al.* (212) studied the distribution of angiopoietins expressed in various limb muscles of rats and demonstrated that both Ang-1 and Ang-2 are expressed at relatively higher levels in the soleus muscle as compared to the white gastrocnemius muscle. This result is not surprising since soleus muscles have greater capillary density than do white gastrocnemius muscles. An interesting aspect of the study is that muscle expressions of Ang-2, and to a lesser extent those of Tie-2, increase significantly and transiently in both muscle types in response to twenty-four days of exercise training (212). This suggests that muscle-specific Ang-2 transcription is regulated by exercise-induced biological processes, including mitochondrial mitogenesis. More recent studies on angiopoietin expression in skeletal muscles have confirmed that Ang-1 and Ang-2 expressions increase in response to exercise training (123, 212, 365).

To date, there are only two studies that have addressed functional effects of angiopoietins, in particular Ang-1, on skeletal muscles. An early study by Dallabrida *et al.*, published in 2005, focused on pro-survival effects of Ang-1 on both cardiac and skeletal myoblasts in culture (65). In that study, human skeletal myoblasts (HSM) and C2C12 cells, an immortalized cell line derived from murine satellite cells, were used. Myoblasts were cultured in the absence of serum on plates coated with different extracellular matrices, including human Ang-1 and Ang-2 (human and mouse Ang-1 and Ang-2 have 97% and 85% homology, respectively). Under these conditions, both

cell types adhered to Ang-1 and Ang-2 in a dose-dependent manner. However, after seven days, cells in the Ang-1 well alone exhibited the highest survival, and where evenly spread on the surface of the plate, were morphologically healthy. Although the authors were able to detect mRNA of Ang-1 in both HSM and C2C12 cells and Ang-2 only in the latter, they were unable to detect Tie-2 receptor mRNA and protein levels in these cells.

To investigate whether or not angiopoietins signal through integrin ligation, they used general and specific inhibitors of integrins, including neutralizing antibodies. They identified α_6 and β_3 as key integrins that mediate adhesion of C2C12 cells to Ang-1 and Ang-2 proteins, and α_2, α_5, α_v, and β_4 to be those that mediated HSM cell adhesion. They also measured caspase 3 activities in serum-starved taxol-treated cells and reported that both Ang-1 and Ang-2 matrices exert important anti-apoptotic effects in both cell types and that these anti-apoptotic effects are mediated through activation of the PI-3 kinase/AKT and ERK1/2 pathways (65).

More recently, Abou-Khalil *et al.* (5) studied angiopoietin/Tie-2 expression and function in cultured human primary skeletal muscle satellite cells and reported that Ang-1 expression declines during differentiation of cells into myotubes but is significantly enhanced in a subpopulation of cells known as regenerating cells (RC). In addition, they were able to detect Tie-2 protein expression and demonstrated significant upregulation of receptors during differentiation into myotubes (5). More importantly, Abou-Khalil *et al.* (5) reported that silencing Tie-2 receptors with siRNA oligos reduces the RC population within a pool of satellite cells, decreases Pax7 expression, and significantly increases MyoD and p57 expressions. Silencing of Tie-2 receptors also causes increases in satellite cell proliferation, myogenin

expression, and myotube formation (5). Similarly, exposure of satellite cells to Ang-1 causes significant decreases in proliferation, which is coupled to reduced apoptosis. On the basis of these results, Abou-Khalil *et al*. (5) concluded that Ang-1 is normally produced by vascular smooth muscles and fibroblasts in skeletal muscles and that Ang-1 acts on a subpopulation of satellite cells that express Tie-2 receptors. They also speculated that this endogenous Ang-1/Tie-2 receptor system helps in the regeneration of satellite cells by maintaining a normal-sized pool of RCs.

Although the Dallabrida *et al*. and Abou-Khalil *et al*. studies have revealed important roles for Ang-1, Ang-2, and Tie-2 in maintaining survival, adhesion, and quiescence of skeletal muscle satellite cells, very important questions regarding their functionality in skeletal muscle biology remain unanswered. For example, functional roles of angiopoietins and Tie-2 in cell differentiation and myotube maturation have not been investigated. Therefore, in Chapter 3 I address the role of Ang-2 in satellite cell proliferation, migration, and differentiation. In Chapter 4 I address the roles of Ang-1 and Tie-2 in the same biological processes. Moreover, the roles of Ang-1, Ang-2, and Tie-2 in enhancing repair of injured muscles in *in vivo* settings are unknown. Therefore, in Chapter 4 I address their functional importance to skeletal muscle regeneration using an *in vivo* model of muscle injury.

1.2 The Skeletal Muscle System

1.2.1 Basic structure

Muscle (n.): derived from the Latin word "*musculus*", meaning "little mouse".

The word was chosen by the Romans who found a resemblance between the movement of muscle under the skin and that of a little mouse running. Skeletal muscles can be considered the largest organ in the human body, and account for 40–50% of total body weight in men and 25–35% in women. Muscle cells have the ability to conduct action potentials along their membrane surfaces and to translate this electrical signal into a mechanical contraction, enabling cells to perform work. Skeletal muscle accounts for approximately 18% of the body's energy consumption rate (basal metabolic rate) at rest. Only 25% of total energy consumed by muscles is used for work, the remaining 75% is released as heat, hence creating an important source of body heat.

The building blocks of skeletal muscles are groups of myofibres bundled within the perimysium. Myofibres are formed by the fusion of skeletal muscle satellite cells known as myoblasts. They are long multinucleated syncytia with post-mitotic myonuclei located at the periphery and pressed against the sarcolemma (plasma membrane in skeletal muscle). The cytoplasm, known as sarcoplasm, contains mitochondria, the sarcoplasmic reticulum, and other cellular organelles (Figure 1.1). The basic functional units of a muscle fibre are sarcomeres, which are repetitive myofibrils of actin and myosin. Myofibres are classified according to their contractile properties, ranging from slow-twitch oxidative to fast-twitch glycolytic fibres. It is the proportion of each fibre type that determines the overall contractile property of a muscle. Satellite cells, which form a population of undifferentiated mononuclear myogenic cells, are closely associated with the basal lamina of the cell and can be distinguished from myonuclei by an abundance of heterochromatin, an indication of mitotic quiescence.

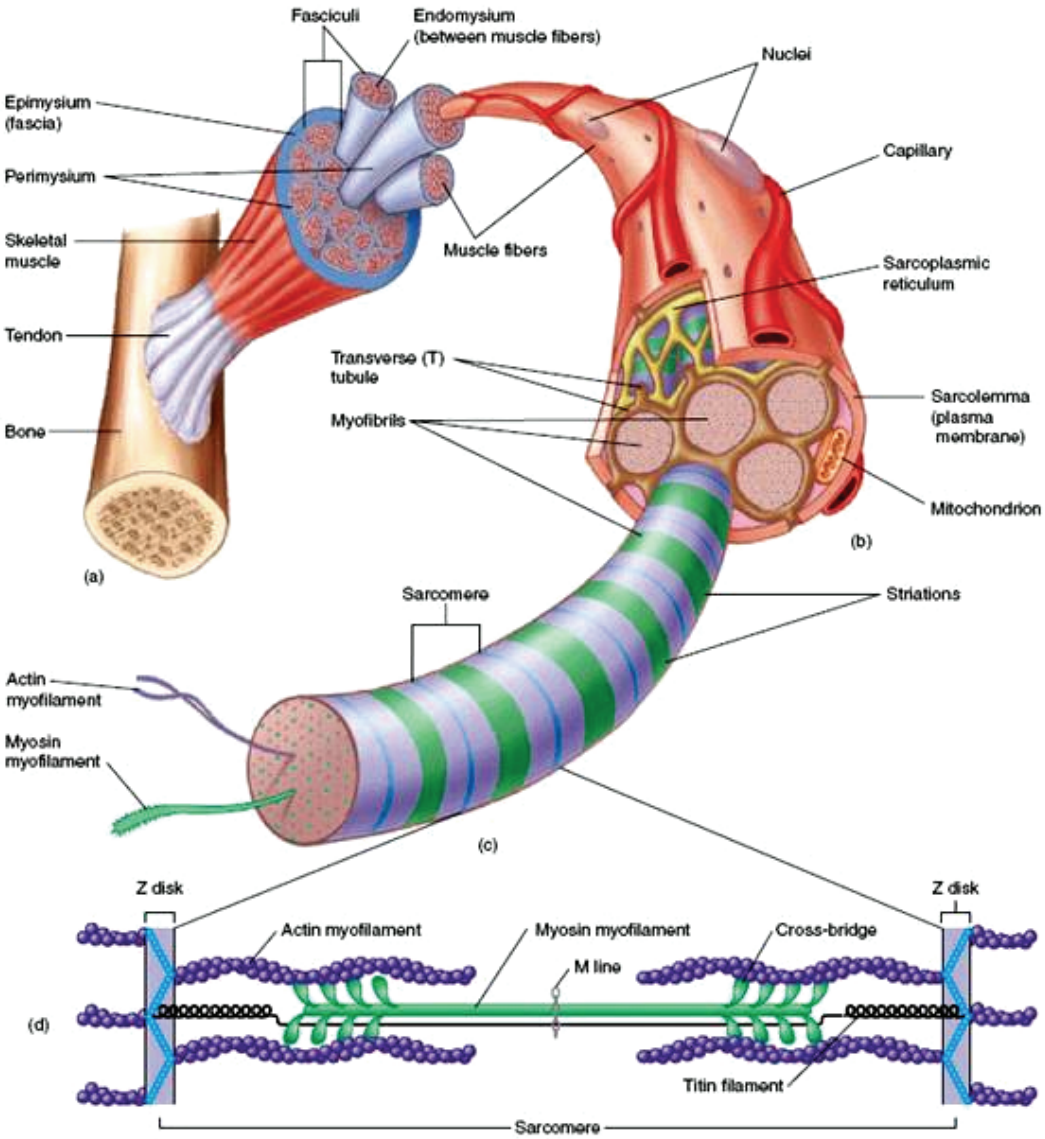

Figure 1.1. Morphological characteristics of adult mammalian skeletal muscle.

1.2.2 Muscle satellite cells

Studies involving skeletal muscle began to appear in the mid-nineteenth century. A set of studies carried out in rodents and birds by Studitsky in the 1950s and 1960s demonstrated that regeneration capacity is relatively high, so that replacing a muscle with its minced form allows regeneration to take place and a fully functional muscle forms (332). Before the development of myoblast cultures, the regeneration process was thought to be amitotic. *In vitro* experiments demonstrated that myoblasts are able to fuse and form large multinucleated skeletal muscle fibres (60), yet researchers were unable to determine the source of myoblasts *in vivo*. In 1961, using electron microscopy to study muscle fibres in frogs, Mauro detected mononucleated cells tightly associated with fibres between the plasma membrane and the basal membrane and named them satellite cells, due to their anatomical position in the fibre (227). The existence of satellite cells was further confirmed by a study showing them to be osmotically independent of underlying myofibres, that is, there was no connection between satellite cell cytoplasm and muscle fibres (245). Using tritiated thymidine, satellite cells were shown to undergo mitosis and give rise to myonuclei necessary for post-natal muscle growth and regeneration (242, 269, 315). A major breakthrough in the field of satellite cells came following the description of the myogenic regulatory factor family (MyoD, Mrf4, Myf5, and myogenin) in the late 1980s. Since myoblasts derived from satellite cells were shown to express these muscle-specific transcription factors, their role as post-natal skeletal muscle stem cells was demonstrated at the molecular level (121, 369). Upon activation, satellite cells not only give rise to myonuclei, but also renew a pool of satellite cells by asymmetric division (18, 243).

1.2.3 Molecular regulation of satellite cell function

During embryogenesis, pair-box family transcription factors (Pax1 to Pax9) are important regulators of development and differentiation of many cell lineages (223). Satellite cells in adult muscle are known to originate from $Pax3^{+}/Pax7^{+}$ satellite cells that stem from the embryonic somite (172, 282). Although Pax3 and Pax7 proteins are structurally similar and bind similar sequence-specific DNA elements, analyzing null mutations in mice have proven that they have non-redundant roles in myogenesis (172, 300, 310, 311). Mice lacking a functional Pax3 gene (known as splotch mice) do not survive to term and due to impaired migration of Pax3-expressing cells from the somite, fail to form limb muscles (357, 357). Pax7 is specifically expressed in satellite cells in adult muscles, daughter myogenic precursor cells, and also in primary myoblast cultures. In $Pax7^{-/-}$ mice, no satellite cells are detectable in skeletal muscle (266, 310), however, in these mice, muscles are smaller in size, fibre diameters are reduced, and contain only half the normal number of nuclei (196).

Although some studies suggest the presence of non-satellite cell muscle stem cells, under physiological conditions satellite cells are considered to be primary contributors to growth and regeneration of skeletal muscle (264). In adult rodent muscles, satellite cells account for 5-10% of myofibre nuclei, which are normally mitotically quiescent (Figures 1.2 and 1.3)(51, 127, 307). The contribution of satellite cells to skeletal muscle hypertrophy and regeneration begins when satellite cells overcome the G0-G1 block and begin to proliferate. In injured muscles, first division

of satellite cells has been shown to occur at approximately thirty hours post-injury (230). Co-expression of MyoD with Pax7 is one of the earliest signs of satellite cell activation, occurring within hours of stimulation, both *in vitro* and *in vivo* (285, 381). One of the main activators of quiescent satellite cells is HGF, through activation of C-met receptors on these cells. HGF is not only involved in the activation of satellite cells, but also promotes proliferation, alongside other growth factors such as insulin-like growth factors (IGFs) and fibroblast growth factors (FGF) (345).

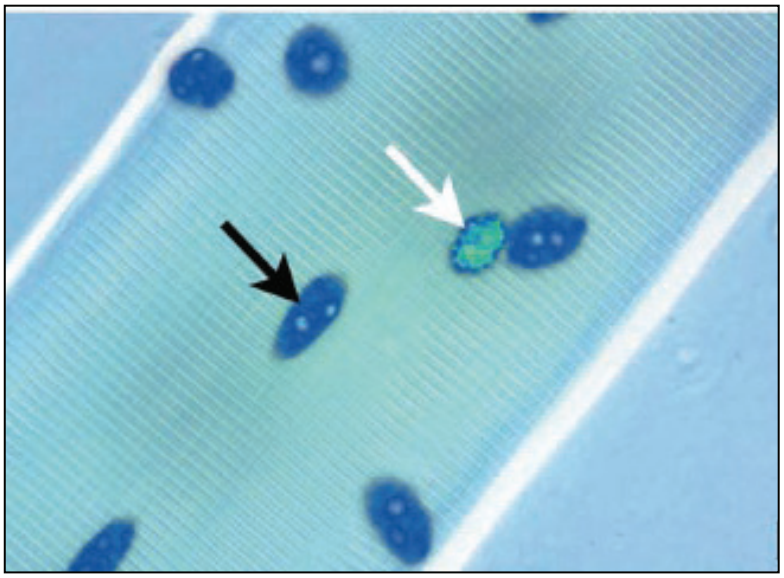

Figure 1.2. Muscle satellite cells on myofibres isolated by mild enzymatic digestion and identified immunocytochemically by high levels of Pax7 (white arrow) as compared to myonuclei (black arrow) (51).

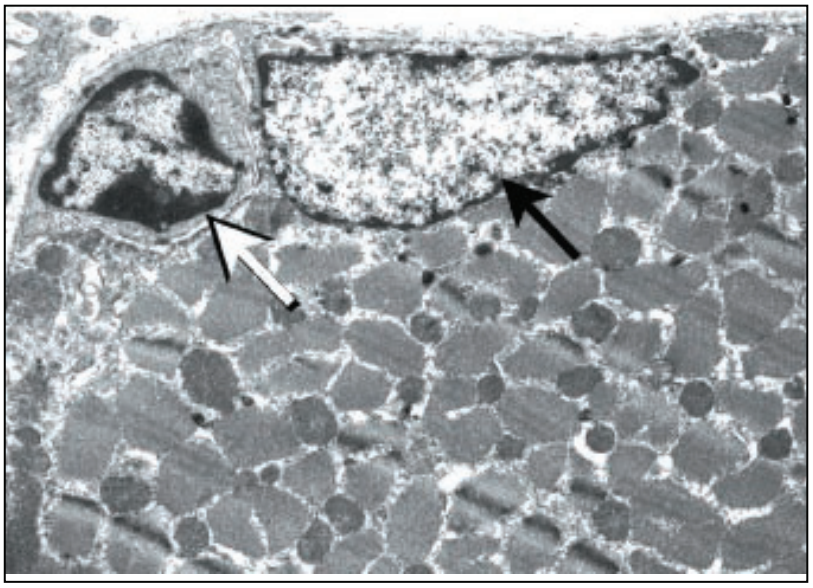

Figure 1.3. Muscle satellite cell nuclei (white arrow) as distinguished from myonuclei (black arrow) by abundant heterochromatin, an indication of mitotic quiescence (51).

1.2.4 Skeletal Muscle Injury and Regeneration

Skeletal muscles are susceptible to direct injury as a result of trauma or indirectly as a result of pathological conditions like sepsis and chronic obstructive pulmonary disease (COPD). These injuries, if not repaired, may lead to the loss of locomotive efficiency, muscle mass, and even death in diseases like Duchenne muscular dystrophy (DMD), where chronic degradation of muscle fibres leads to failure of the respiratory muscles. Although adult skeletal muscle has slow turnover of myonulei (1-2% in rats), it has rapid and extensive regeneration capacity when injured (304). The muscle regeneration process consists of overlapping phases of degeneration, inflammation, and regeneration.

Muscle injury entails disruption of muscle fibres, plasma membranes and basal laminae. As a result, leakage of calcium and activation of proteases occurs that, in turn, lead to necrosis of fibres by autodigestion (160). Intact fibres are protected from the degeneration process by the formation of "contraction zones", which are sarcolemmal boundaries that isolate sites of injury (151, 261). Degeneration of injured muscle is aggravated by local swelling, formation of hematomas, and infiltration of macrophages and T-lymphocytes to sites of injury. Neutrophils are the first inflammatory cells that are present at sites of injury in high numbers, one to six hours after exercise or myotoxin-induced injury (99, 255). They remove debris by releasing proteases that degrade damaged fibres (322). In a study where mice received neutrophil antisera thirty-six hours prior to muscle injury, the regenerative capacity of muscle was compromised due to the presence of necrotic debris (348). Neutrophils also release cytokines that signal monocytes and promote their transformation into macrophages. Approximately forty-eight hours post-injury,

macrophages become the predominant inflammatory cells at sites of injury, where they phagocytose cellular debris and promote muscle regeneration by activating myogenic cells (204, 232, 255, 355).

In addition to phagocytosing, macrophages release cytokines such as TNFα, interleukin-6, and interleukin-8 that increase vascular permeability at sites of injury and result in more inflammation. Macrophages have also been shown to release growth factors such as heparin-binding EGF-like growth factor (HB-EGF), which is involved in promoting terminally differentiated myotubes. This growth factor also supports survival of muscle cells against oxidative stress (143). The fact that intensive physical exercise can stimulate peritoneal macrophages is an indication that inflammatory responses are not limited to damaged muscle in the case of muscle injury (93, 214, 374). The inflammatory phase of muscle degeneration is followed by activation of the muscle repair process. Reduced muscle regenerative capacity in studies where irradiation or anti-mitotic reagents such as colchicine are used highlights the necessity of cellular proliferation in the regeneration process (273, 366). The contribution of dividing cells to regenerating myofibres has also been shown by numerous radiolabeling experiments. Resultant myogenic cells differentiate and fuse to existing damaged fibres to repair them or form new myofibres by fusing to one another (323, 324). Mature skeletal muscle fibres contain post-mitotic nuclei and are, therefore, unable to divide and repair damaged muscle.

Satellite cells are the main contributors to muscle regeneration and their absence has been shown to effect a dramatic reduction in the regenerative capacity of muscle (197). Upon injury, satellite cells are activated and begin to proliferate (308).

Following several rounds of proliferation, the majority of satellite cells differentiate, fuse to existing damaged fibres, or form new fibres. Newly-formed myofibres express embryonic forms of myosin heavy chain (MHC), which is an indication of *de novo* fibre formation. Due to high protein synthesis, these newly-formed myofibres are mainly basophilic and can be identified histologically by their small calibre and centrally located myonuclei (128, 370). Longitudinal sections of newly-regenerated fibres indicate that cell fusion is focal to sites of injury (32). Upon completion of myogenic cell fusion, newly-formed myofibres increase in size and myonuclei are pushed to the periphery of muscle fibres, at which point regenerated fibres are morphologically and functionally indistinguishable from intact fibres.

1.2.5 Muscle regeneration: Transcriptional cascades

Main advances in the field of muscle development can be attributed to the development of *in vitro* models where events related to skeletal muscle differentiation can be reproduced. Cultured skeletal myoblasts are immortalized muscle cells isolated from embryos that are able to proliferate *in vitro* in the presence fetal bovine serum. They are bound to their myogenic lineage and express skeletal muscle-specific structural genes only when they are forced to exit the cell cycle. Irreversible withdrawal from the cell cycle is followed by myoblast differentiation and fusion to form multinucleated myotubes. These muscle structures express a wide variety of muscle-specific genes that are necessary for the formation of muscle contractile and metabolic properties.

MyoD was the first myogenic regulatory gene to be isolated from myoblasts. This was achieved by cloning mRNA that was expressed in myoblasts but not in 10T1/2 fibroblasts (58). In MyoD deficient mice, muscle recovery takes place at a

slower rate than in non-deficient mice (231). MyoD is not expressed in quiescent satellite cells, but once they leave the basal laminae and begin to proliferate, MyoD is activated. In the absence of MyoD, activated satellite cells exhibit reduced capacity to stop proliferation and initiate differentiation into muscle fibres (61). Subsequently, three related genes, myogenin, Myf5, and Mrf4 were discovered (268). All four genes, known as the MyoD family, are undetectable in smooth or cardiac muscle; they are exclusive to skeletal muscle. They are known as effectors of a master switch that regulate synchronized activation and expression of muscle-specific genes in cells. Their activation suffices to lead even terminally differentiated non-muscle cells into the myogenic conversion process (58).

The transcription factor myogenin, in addition to inducing skeletal muscle-specific genes, ensures the process of cell cycle withdrawal by activating transcription of genes such as $p21^{kip}$ (37). In satellite cells, myogenin is expressed as the differentiation process begins (37). Mice lacking myogenin die perinatally due to the absence of myoblast differentiation and the complete absence of myofibres (138, 247). Although the absence of myogenin in the post-natal period does not affect growth and development of muscle mass, survival is compromised and survivors are smaller by 30%, suggesting a disruption of some muscle autocrine function (186).

Mrf4, along with MyoD and myogenin, is expressed in newly-formed myotubes and in adult muscle fibres; it is not expressed in satellite cells (389). Mutant Mrf4 mice are viable, but suffer from developmental defects of some trunk muscles, including the thoracic muscles (364). In these mice, despite increased expressions of other myogenic factors, Na^+ channel gene expressions are selectively downregulated in both neurotransmitter junctions and the surface membrane (351).

Like MyoD, Myf5 is also expressed in proliferating satellite cells. Absence of Myf5 has been shown to cause growth defects and premature differentiation (239).

Pax genes are a family of transcription factors that have crucial roles in organogenesis and tissue specification during embryogenesis. Pax3 and Pax7 have been extensively studied in the context of myogenic precursor cells. Pax proteins play an important role in the entrance of satellite cells into the myogenic program. In dominant-negative Pax3 and Pax7 cultured satellite cells, MyoD expression is inhibited (281). Pax gene expression is downregulated once satellite cells begin to differentiate, and those that maintain high levels of Pax7 are critical to repopulation of the satellite cell pool (386).

Coordinated expression of the transcriptional cascade involved in muscle regeneration has been explored *in vivo* by several investigators. In a study completed by Zhao *et al*, where regeneration was induced in mouse gastrocnemius muscle by injection of cardiotoxin, a gene expression profile of twenty-seven time points (day 0 to day 40), covering both degeneration and regeneration, was produced using an Affymetrix U74Av2 mouse gene expression array (387). Clustering analysis of this data revealed inductions of genes related to three processes that are involved in muscle degeneration and regeneration (387). At early time points (day 0-day 2) muscle structure genes were downregulated while inflammation-related genes were induced. By day 3-day 3.5 significant inductions of myogenic related genes appeared, including myogenin, MyoD, and embryonic MHC, and, finally, at later time points, muscle structural genes such as myosin and actin were expressed at normal levels (387, 388). On day 1-day 3 of muscle regeneration, cardiotoxin-induced muscle regeneration in tibialis anterior (TA) muscles exhibited inductions of

genes involved in cell cycle control and DNA replication. The time point at which myogenic regulators and genes involved in differentiation of proliferating cells were induced was day 3-day 5(383).

1.2.6 Satellite cell myogenesis: Roles of growth factors

Post-natal myogenesis by satellite cells is comprised of different phases that include exit from quiescence (248), proliferation (173), exit from the cell cycle and differentiation (205), migration (31), and fusion to existing myofibres (144). Each step has been shown to be regulated by various extracellular signals including growth factors. Three main families of growth factors have been extensively studied in this context. They include HGF, FGFs, and TGFβ.

HGF, previously known as scatter factor, is expressed in both intact and regenerating muscle and functions by activating a heterodimeric RTK named c-Met (35). Expression levels of HGF in injured muscles are highest during the early stages of regeneration; the magnitude of its expression is proportional to the degree of injury (158, 339, 345). The presence of c-Met receptors in both satellite cells and proliferating myoblasts enables them to respond to the pro-proliferative signal of HGF produced by muscle cells and the spleen (173). HGF is produced as a biologically inactive pro-HGF and binds to extracellular matrix molecules until it is cleaved and converted into its active form (249). In response to myofibre stretch or injury, the basal lamina releases NOS, which, in turn, produces NO. Activation of matrix metalloproteinases by NO leads to formation of the active form of HGF, which promotes activation and proliferation of satellite cells (346, 382). Another mechanism through which HGF promotes myogenesis in an injured muscle is by promoting satellite cell migration to sites of injury (31, 338).

Fibroblast growth factors, a large family of related polypeptide growth factors, are known to act as mitogens, differentiation factors, and regulators of programmed cell death during development, post-natal life, and in pathological conditions (256, 257, 340). FGFs require a dual receptor system to activate signal transduction pathways, which include tyrosine kinase transmembrane FGF receptors (FGFR1 through FGFR4) and their co-receptors, cell surface heparin sulphate proteoglycans (HSPGs) (90, 258). Certain FGFR members can be detected in both adult muscle tissue *in vivo* and in cultured myogenic cells, *in vitro*. Satellite cells express mainly FGFR1 and FGFR4 (173). The presence of FGFs has been shown to have differential effects on myogenic cells. Certain FGFs promote proliferation of myogenic cells and suppress or delay differentiation into myotubes, while others induce proliferation with no effect on the differentiation process (73, 190, 380). In the context of satellite cells, FGF6 is of special interest. This growth factor is highly expressed in developing muscle during embryogenesis and in both healthy and regenerating adult muscle, making it an effective promoter of satellite cell proliferation and skeletal muscle maintenance (13, 64). As important as the role of FGF6 is in myogenesis, studies completed with FGF6 knockout mice have revealed an important role for FGF2 in that it compensates for a lack of FGF6. Thus, different FGFs may be able to compensate for each other in the *in vivo* myogenesis process (250).

The transforming growth factor beta (TGFβ) super-family is comprised of more than forty members (318). A member of this family that has been of interest in the context of myogenesis is myostatin. Myostatin maintains proper muscle mass during development and adult life by negatively regulating the growth of skeletal

muscle (48, 166). Its loss leads to increased fibre size and number, which results in a phenotype of significant muscle overgrowth (251, 306). Negative effects of myostatin on proliferation and differentiation of myoblasts *in vitro* have been shown to be mediated through induction of cell cycle arrest and downregulation of myogenic differentiation factors, respectively (165, 284, 350). In the context of satellite cells, it has been suggested that myostatin inhibition of G_1 to S progression is designed to maintain these cells in the quiescent state. This is evidenced by the observation that myostatin null mice have higher numbers of proliferating satellite cells as compared to wild type mice (229). Furthermore, the inverse relationship between the number of satellite cells in a muscle and its myostatin levels is has also been shown in various muscle fibre types; slow-twitch muscle fibres, which naturally contain lower levels of myostatin, possess more satellite cells than do fast-twitch muscle fibres (368).

1.2.7 Animal models of muscle regeneration

Several well-established animal models exist in which investigators have explored molecular mechanisms and therapeutic potentials of various factors. These models involve either partial or complete degeneration of muscle fibres. Each will be addressed in turn.

1.2.7.1 Partial degeneration models:

Crushing injury model: This model involves inflicting mechanical trauma to a muscle using a mechanical apparatus, such as dropping a mass of a certain weight from a certain height onto the muscle (235). It has two major disadvantages, namely, poor reproducibility and the inability of certain fibre types to regenerate in response to this type of injury, as compared to other injury models. Indeed, several reports have confirmed that fast-twitch and slow-twitch muscle fibres possess different

regenerative properties, with slow-twitch fibres muscles, such as those in the soleus, having reduced regenerative capacity in response to crush injury as compared to their capacity to regenerate in response to toxin-induced injury (27, 100).

Heat or cold injury: The main purpose of designing thermal injury models is to create situations where human muscles are damaged. Freeze injuries can also be used as models of injury where no surviving cells remain, although muscle integrity remains intact. This is a perfect scenario for situations where the contribution of existing exogenous cells or of implanted cells are to be assessed. The most common way of producing injury in this model is by placing hot or cold pieces of metal directly onto muscle or skin (241, 356).

1.2.7.2 Complete or near-complete degeneration models:

Minced muscle: In this model, a muscle of interest is removed, minced into small pieces and implanted back into the muscle bed. This is by nature an ischemia model where the destruction of all muscle fibres is certain. In this model, all stages of muscle regeneration, including reinnervation, revascularization and activation of satellite cells to maturation into myotubes can be histologically documented in a single regenerating muscle. A major disadvantage of this model, especially in rats, is the formation of connective tissue that interferes with muscle regeneration. Also, the size of the muscle that is minced largely determines the success of this model; in larger muscles, muscle regeneration is limited by the rate of revascularization (46).

Free muscle grafting: In this ischemic necrosis model, a muscle is completely removed from its bed, replaced orthotopically, and cut tendons are reconnected, thus allowing re-innervation and re-vascularization to take place spontaneously. The overall success of this model depends on the completeness of reinnervation rather

than revascularization; if motor nerves to the muscle are left intact, the muscle recovers to approximately 100% of its original mass and 90% of its maximum contractile force, while in the case of severed nerves, maximum titanic force can only recover to approximately 33% of control levels. Unlike the minced muscle model, in this model very little scar tissue is formed. Its usage is limited by the size of the muscle of interest and tends to be unsuccessful when applied to large muscles (47).

Cross-transplantation: This model is a very good candidate for studies where the effects of environmental factors versus intrinsic qualities of the muscle regeneration process are of interest (125).

Injection of myotoxic agents: Myotoxic agents that are used include, but are not limited to, local anaesthetics and toxins. Most local anaesthetics are myotoxic (40), however bupivacaine (Marcaine) is the most commonly used agent in experimental settings. A major advantage of Marcaine use is that with a single injection, non-ischemic injuries are induced in a large number of muscle fibres (29). The most common muscle used with this model is the tibialis anterior muscle. One disadvantage of this model is that some myofibres might be spared and left uninjured. This could be a problem in gross analysis; where muscle is histologically studied, the presence of some normal fibres can be used an internal control.

Toxins, in particular venom myotoxins, are the most widely used agents for inducing muscle fibre injury. Myotoxins can be placed into three groups: cobra venom myotoxins, which are 60-65 amino acid polypeptides; phospholipase A_2 toxins, from a wide variety of snakes; and cardiotoxins, which are small polypeptides from new world vipers. The types of venoms used in inducing myofibre injury are not

limited to snake venom. Venom from tarantulas and bees can also induce muscle necrosis (124, 260).

Cardiotoxins (also known as cytotoxins) are single chain small polypeptides that are related to nAChR-binding α-neurotoxins (85). These toxins form pores in the sarcolemma of skeletal muscle that eventually result in its polarization and degradation (Figure 1.4) (101, 124). The basal lamina remains intact, creating a scaffold where the regeneration process takes place. The main consequence of membrane leakiness is rapid and prominent calcium influx, which activates the contractile machinery and results in hypercontraction of the myofibrillar apparatus (136). Hypercontraction is the overcontraction of some sarcomeres, with consequent overstretching of others, which results in alternating regions with either clumped myolfilaments or no myofilaments at all (213). Mechanical effects of hypercontraction cause further plasma membrane damage (81). Hypercontraction is not the only consequence of Ca^{2+} release; certain cytosolic calcium-dependent proteases like m- and μ-calpains are also activated. These proteases are responsible for early myofibrillar degradation that further destabilizes muscle integrity. Release of Ca^{2+} also leads to mitochondrial calcium overload, alterations in the mitochondrial membrane, and loss of control of its volume (375).

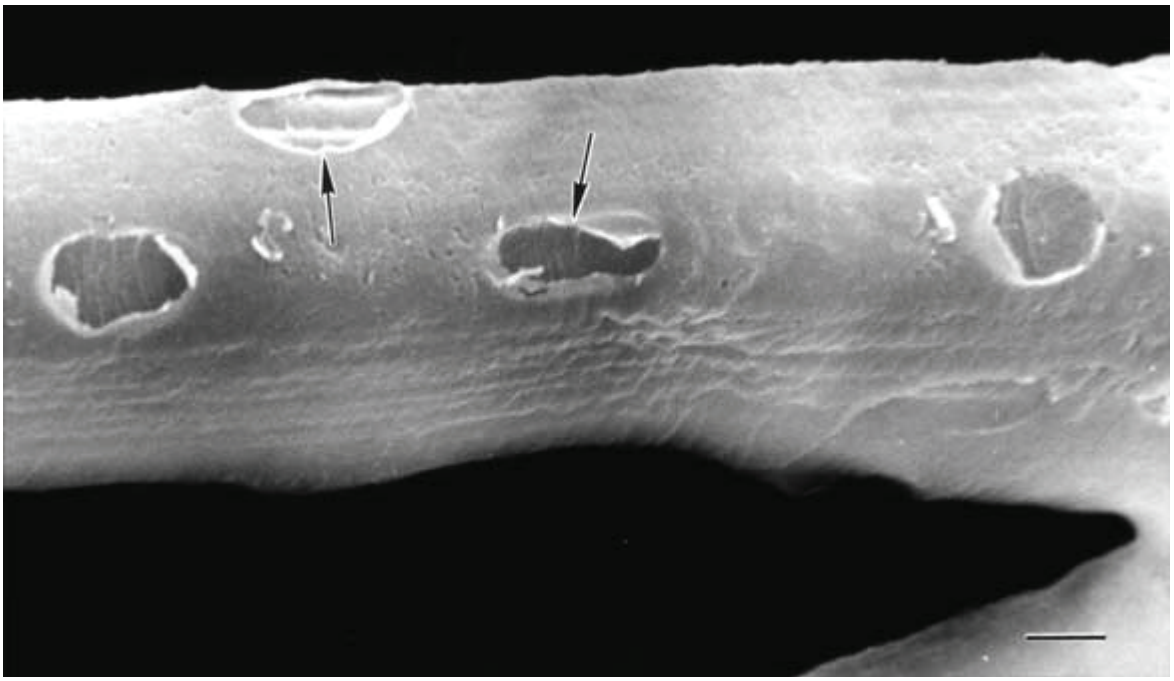

Figure 1.4: Scanning electron micrograph of a portion of a skeletal muscle cell from mouse soleus muscle 3hr after intramuscular injection of 10mg of myotoxin from *Agkistrodon contortrix laticinctus* venom, Note the presence of various lesions in the plasma membrane (arrows). Bar represents 10mm (124).

1.2.8 Skeletal muscle regeneration: Roles of vascular endothelial growth factor

Vascular endothelial growth factor-A (VEGF-A) is one of the key regulators of blood vessel formation during embryogenesis and a strong inducer of neovascularization in the course of adult life. There are three main receptors involved in the mediation of biological effects of the VEGF family of growth factors - VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1) and VEGFR-3 (Flt-4). VEGF-A functions mainly through VEGFR-2 in the context of angiogenesis and induces vasodilatation, enhances vascular permeability, and promotes proliferation, migration, and survival in endothelial cells (94, 384). It was originally described to be endothelial-specific, but recent studies have shown that the effects of VEGF-A extend to other cell types.

In vivo experiments in normal skeletal muscles have revealed that expressions of VEGF, VEGFR-1, and VEGFR-2 receptors are limited to quiescent satellite cells and vascular structure (111). Furthermore, it has been shown that proliferating primary murine skeletal myoblasts and C2C12 cells express VEGF, VEGFR-1, and VEGFR-2; the latter show constitutive tyrosine phosphorylation (111). In an ischemic model of muscle injury induced by ligation of the femoral artery, VEGF receptors were detected in both activated satellite cells and proliferating myogenic cells; VEGF protein was observed in newly-formed myofibres (111). Newly-formed myofibres, distinguished by their central nuclei and small size, exhibited diffuse patterns of VEGFR-1 and VEGFR-2 expression. Upon completion of muscle regeneration (fourteen days post-injury), receptor expression levels were similar to those of uninjured muscle. These findings suggest that the VEGF/VEGF receptor axis is

significantly induced within skeletal muscle satellite cells and newly-formed fibres during the early phases of ischemia-induced muscle regeneration.

One of the important stimuli that lead to induction of muscle-derived VEGF production appears to be hypoxia. Exposure of C2C12 myoblasts to hypoxia *in vitro* has been shown to induce VEGF levels by 5-fold, while neutralization of VEGFR2 by a specific antibody triggers a significant increase in apoptosis (111). These observations suggest that enhanced production of VEGF in response to hypoxia has an anti-apoptotic effect on hypoxic muscle cells in culture.

Contradictory results have been published regarding changes in VEGF and VEGF receptor expression in skeletal muscle satellite cells during differentiation into mature myotubes. Germani *et al*. have reported that upon differentiation of primary murine skeletal and C2C12 myoblasts, expressions of VEGF, VEGR-1 and VEGFR-2 decline significantly as MHC expression rises (111). They suggest that the differentiation program has an inhibitory influence on muscle-derived VGEF/VEGF receptor expression. The results of Germani *et al.* contradict those of Arsic *et al.* (14) who have shown that expressions of both VEGFR-1 and VEGFR-2 increase significantly when differentiation of C2C12 myoblasts is induced and remains high throughout the differentiation process (14). Arsic *et al.* (344) also found that supplementing differentiation media with VEGF decreases proliferation while increasing both the diameter of myotubes and the number of multinucleated cells within myotubes. These results suggest that VEGF promotes both differentiation and hypertrophy of muscle satellite cells. Reasons behind these contradictory observations remain unclear. One possibility is the use of different experimental approaches and indices to evaluate the muscle differentiation program.

In normal skeletal muscle fibres, studies have shown that enhancing VEGF expression may induce alterations both in the vasculature and in muscle fibre themselves. For instance, when myoblasts constitutively expressing VEGF-A are implanted in a non-ischemic muscle, a significant increase in recruitment of endothelial cells and macrophages is observed (327, 328). This initial response is followed by overgrowth of capillary-like blood vessels and the formation of hemangiomas at the site of implantation (327, 328). In a separate study, Arsic *et al.* demonstrated that injection of adeno-associated viruses expressing VEGF (AAV-VEGF) into normal TA muscle induces a 5% increase in the number of muscle fibres containing central nuclei, with almost 20% of fibres being hypertrophic one month post-injection (14). These results suggest that VEGF overexpression triggers hypertrophy of normal muscle fibres.

Therapeutic effectiveness of the VEGF/VEGF receptor axis in skeletal muscle regeneration has been studied in different models of muscle injury. In an ischemic model of muscle injury, injection of adenoviruses expressing VEGF cDNA before induction of limb muscle ischemia triggered a significant improvement in survival of endothelial cells, smooth muscle cells, and skeletal muscle satellite cells (111). In addition to improving cell survival, the influence of the VEGF/VEGF receptor axis on muscle regeneration is believed to be mediated in part through enhancement of migration to sites of injury of not only endothelial cells but muscle satellite cells (111). This conclusion is supported by the fact that skeletal muscle satellite cells that express both endothelial and myogenic markers, including VEGFR-2, are present in the embryonic dorsal aorta of mouse and are involved in post-natal growth and regeneration of skeletal muscle (71). It is possible, therefore, that in the case of

muscle injury, the processes of muscle regeneration and angiogenesis are coordinated by common molecular pathways.

Another model of muscle injury is induced by glycerol injection. This triggers destabilization of the cytoplasmic membrane followed by cell death. Administration of AAV-VEGF prior to or a few days following initiation of muscle injury has been shown to reduce the size of injury and to shorten recovery time (14). An interesting observation in that study is that while improvement in muscle regenerative capacity is dependent on the dosage of AAV-VEGF viruses, the number of newly-formed arterioles (index of angiogenesis) increased significantly irrespective of the AAV-VEGF dose, suggesting that the effects of VEGF therapy on muscle regeneration are independent from its pro-angiogenic role.

VEGF therapy has also been shown to be very effective in necrotic muscle injury models where snake venom is injected within a given skeletal muscle. For example, in a cardiotoxin-induced mouse TA muscle injury model, injection of AAV-VEGF after induction of injury results in significant reduction of injured areas, from 40% of total cross-sectional area in untreated muscles to 25% in the AAV-VEGF group (14). Higher doses of AAV-VEGF induced complete recovery of muscle twenty days post-injury, while recovery of untreated groups occurred after approximately forty days. The observation that injection of AAV expressing mouse placental growth factor (PlGF), a member of the VEGF family that specifically activates VEGFR-1, did not improve the regeneration process in cardiotoxin-induced injury suggests that VEGFR-2 is the main transducer of VEGF signals in injured muscle (14).

The cellular pathways through which the VEGF/VEGF receptor axis regulates survival and differentiation of skeletal muscle satellite cells remain under investigation, however, there is indirect evidence suggesting that both the PI-3 kinase/AKT and MAPK pathways are involved. In endothelial cells, activation of VEGFR-2 by VEGF promotes survival, proliferation, and differentiation, mainly through PI-3 kinase/AKT and MAPKs (110). In skeletal satellite cells, activation of the PI-3 kinase/AKT pathway inhibits apoptosis during differentiation and promotes myotube hypertrophy (34, 105). In an *in vivo* model, where muscle fibres were transduced by constitutively active AKT, the hypertrophic response of muscle fibres to activation of the pathway was shown to be accompanied by increased levels of VEGF (343). The MAPK signalling pathways, and in particular the ERK1/2 pathway, are known to promote muscle cell differentiation through increasing activation and expression of MyoD protein (120). The activation of this pathway in endothelial cells is well-documented in endothelial cells exposed to VEGF.

Unlike the increasing interest that has been shown in studying the functional significance of the VEGF/VEGF receptor axis in regulating skeletal muscle regeneration, there is no information as yet available regarding the contribution of the angiopoietin/Tie-2 receptor axis to skeletal muscle regeneration. In chapter 4 of this thesis, I address alterations in Ang-1, Ang-2, and Tie-2 receptor expression in skeletal muscle satellite cells, both during the quiescent phase and in response to the differentiation program. I also address changes in Ang-1, Ang-2, and Tie-2 expression during injury and regenerative phases of cardiotoxin-induced TA muscle injury in mice. I also explore therapeutic potentials of increased Ang-1 expression following initiation of TA muscle injury in mice and address mechanisms through

which the angiopoietin/Tie-2 receptor axis regulates skeletal muscle differentiation in both *in vitro* and *in vivo* settings.

1.3 Skeletal Muscle Function in COPD Patients

Chronic obstructive pulmonary disease is a debilitating lung disease characterized by inflammation-induced chronic lung parenchymal destruction and not fully-reversible airflow limitation. The disease affects more than 10% of the population worldwide (38). Predominant symptoms of COPD include chronic productive cough and exertional dyspnea. In addition, a large percentage of COPD patients have extrapulmonary manifestations, including weight loss and cachexia. Other common characteristics include skeletal muscle weakness, heart failure, osteoporosis, ischemic heart disease, stroke, and depression (8). Exertional dyspnea and limitation of exercise capacity in COPD patients have been blamed on augmentation of work of breathing as well as on impairment of gas exchange. However, there is increasing evidence suggesting that skeletal muscle dysfunction is a major cause of exercise limitation and poor quality of life in COPD patients (238).

1.3.1 Structural alterations in limb muscles of COPD patients

The main feature of limb muscle pathology in COPD patients is loss of muscle mass and reduction in cross-sectional area (muscle atrophy){3973, 3975, 3990}. The magnitude of loss of thigh muscle mass is relatively greater than that of whole body weight, indicating a preferential loss of muscle tissue over other body tissues in emaciated COPD patients (88). In addition, several studies have measured proportions of different skeletal muscle fibre types, or their corresponding myosin isoforms, in COPD. Most studies have focused on vastus lateralis (VL) muscles and

document a reduction in the proportion of type I slow-twitch type muscle fibres and a corresponding increase in the proportion of type II fibres in COPD patients, as compared to controls {3992, 4000, 3990}. Whittom *et al.* {3990} have reported that type I fibre proportion declines by 20%, whereas the proportion of type IIb fibres increases by 10%, in limb muscles of patients with severe COPD. In addition, fibre-type shifting towards more glycolytic fibres (type IIb) is associated with decreased muscle oxidative capacity, as shown by significant reductions in cytochrome c oxidase and succinate dehydrogenase (115). It should be emphasized that the above-described changes in fibre-type distribution may differ in skeletal muscles other than the vastus lateralis. For instance, deltoid biopsies of COPD patients exhibit no significant changes in fibre-type proportions and show that activity of citrate synthase is preserved or even increased (109).

1.3.2 Changes in contractility and endurance

COPD-induced alterations in muscle strength (defined as the capacity of muscle to generate force) primarily involve lower limb muscles, with quadriceps femoris muscle strength being 20 to 30% lower in patients with moderate to severe COPD as compared to control subjects (102, 117). It has now been established that the degree of reduced limb muscle strength correlates with severity of the disease process, and low limb muscle strength contributes to poor exercise performance, increased dyspnea, and worsening of quality of life (117, 244). Relatively low limb muscle strength is a powerful predictor of mortality in severe COPD patients (238). In addition to attenuation of muscle strength, many reports have confirmed that endurance (defined as the capacity of muscle to maintain a certain force over time) of limb muscles is attenuated by approximately 30% in patients with moderate COPD

and that poor muscle endurance in these patients correlates positively with physical activity index, FEV1, and resting PaO2 (314, 361). Studies have also shown that COPD-induced decreases in limb muscle endurance are related to reduction of mitochondrial oxidative capacity and to the development of oxidative stress (11). The exact contribution of atrophy to contractility impairment and reduced endurance of limb muscles of COPD patients has been a point of considerable debate. Several reports have found that when limb muscle strength is normalized per cross-sectional area or mass, no differences can be observed between COPD patients and control subjects, suggesting that muscle atrophy is the sole cause of reduced limb muscle strength (30, 86, 87). Debigare *et al.* (75) supported this conclusion by reporting that *in vitro* tetanic tension of VL muscle bundles from COPD patients are similar to those obtained from control subjects when tension is normalized per cross-sectional area. However, there are many investigators who believe that intrinsic abnormalities rather than muscle atrophy may be responsible for poor contractile performance and enhanced fatigability of limb muscles of COPD patients (217, 289, 290).

1.3.3 Adaptations in ventilatory muscles of COPD patients

The degree of skeletal muscle dysfunction in COPD patients is not uniform between muscle groups. Gosselink *et al.* have found that proximal upper limb muscle strength is more impaired than distal upper limb muscle strength in patients with moderate COPD (102). Milder reduction in arm strength than that of leg strength, which is more severely attenuated, has also been described in COPD patients (102). It has been speculated that these differences between various skeletal muscles in COPD patients, which relate to levels of reduction of muscle contractile performance, are related to the pattern of daily muscle recruitment and activation. This is clearly the

case with respect to ventilatory muscles, in particular the diaphragm, which exert different workloads than do limb muscles. Ventilatory muscles are in a chronically overloaded state due to the increased work of breathing brought on by airflow obstruction and hyperinflation. This overloaded state has been linked to distinctly different adaptive changes in the diaphragm as compared to limb muscles. Indeed, the diaphragms of patients with severe COPD have larger proportions of type I fibres (64% of total fibres) as compared to control diaphragms (45% of total fibres) (206). The proportion of type I fibres in the diaphragms of COPD patients correlates negatively with resting FEV1 (% predicted), suggesting that diaphragm fibre-type switching, to more fatigue resistant fibres, is highly dependent on severity of the pulmonary disease process (208). Fibre-type switching toward an increase in proportion of type I fibres has also been observed in other inspiratory muscles, including the parasternal intercostal muscles (207).

1.3.4 Mechanisms of skeletal muscle dysfunction in COPD patients

A set of complex interactions between many factors, both systemic and local, has been implicated in skeletal muscle dysfunction in COPD patients. Systemic factors include inflammation, inactivity, malnutrition, hypoxemia, and smoking. Local factors include oxidative and nitrosative stress of muscle fibres, changes in the balance between protein synthesis and degradation, and impaired vascularization.

1.3.4.1 Inflammation: It has been well established that in addition to having pulmonary inflammation, patients with COPD express several markers of systemic inflammation, including increased circulating leukocytes and elevated plasma levels of pro-inflammatory cytokines such as TNFα, interleukin-6 (IL6), interleukin-8

(IL8), and soluble TNF receptors 55 and 72 (80, 305). Although there is no direct evidence implicating systemic pro-inflammatory cytokines in skeletal muscle dysfunction, there are many observations that support the notion that systemic inflammation is a factor in determining poor contractile performance of skeletal muscles in COPD patients. First, serum TNFα, IL6, and IL8 levels correlate negatively with limb muscle strength (329, 385). Second, pro-inflammatory cytokines in general, and TNFα in particular, can negatively influence skeletal muscle strength and contractile performance. For instance, it is well known that TNFα promotes skeletal muscle wasting through activation of the proteasomal degradation pathway and by induction of apoptosis (45, 200). TNFα may also reduce skeletal muscle oxidative capacity by inhibiting mitochondrial biogenesis (360). In addition, TNFα can directly inhibit skeletal muscle contractility by reducing myofilament protein sensitivity to Ca^{2+} and by enhancing generation of ROS and RNS (280).

1.3.4.2 Inactivity: Several authors have suggested that reduced muscle activity and physical deconditioning are primary causes of reduced muscle strength and atrophy of limb muscles in COPD patients. This argument is based on observations that inactive skeletal muscles and those observed in limb muscles of COPD patients exhibit similar pathological changes, which include reduction of proportion of type I fibres, muscle atrophy, reduction in mitochondrial enzyme capacity and biogenesis, and reduction in capillary density (103, 201, 283). However, there is evidence that changes in physical activity alone are not sufficient to cause limb muscle dysfunction in COPD patients. For instance, the proportion of type I fibres in limb muscles of COPD patients is significantly greater than those observed in response to inactivity in

healthy subjects (11, 115, 271). Moreover, prolonged physical training elicits only a relatively small increase in the proportion of type I fibres in limb muscles of COPD patients as compared to control subjects (219). Finally, Couillard *et al.* (62) studied COPD patients and control subjects with similar levels of daily physical activity and concluded that quadriceps endurance in the former remained significantly lower than in the latter, suggesting that inactivity is not the sole cause of reduced muscle endurance in COPD.

1.3.4.3 Malnutrition**:** Malnutrition that is caused by an imbalance between energy intake and energy expenditure is frequently observed in COPD patients (305). It should be noted that while malnutrition may contribute to peripheral muscle atrophy in COPD, it fails to fully explain changes in fibre-type distribution, mitochondrial dysfunction, loss of capillary density, and increased oxidative stress. Moreover, nutritional supplementation triggers a relatively small improvement in the muscle function of COPD patients, suggesting that nutritional depletion may not be a primary mechanism underlying skeletal muscle dysfunction in the disease (95).

1.3.4.4 Hypoxemia: Hypoxemia elicits significant reductions in skeletal muscle contractile performance, increases in the degree of muscle protein degradation, and substantial attenuation of mitochondrial enzyme activity (44, 142). Hypoxemia-induced reductions in skeletal muscle contractile performance may be mediated through direct inhibitory effects on muscle contractile proteins (316), or indirectly through release of pro-inflammatory cytokines such as TNFα and IL1β (113). The fact that patients with COPD develop chronic hypoxemia has led many investigators to propose that hypoxemia is a main contributing factor to poor skeletal muscle

function in these patients. Although this notion is supported by observations that exercise endurance and high energy metabolite levels, such as those of ATP and creatine phosphate, correlate with arterial PaO_2 in COPD patients (156, 314), direct involvement of relatively low PaO2 in muscle contractility impairment remains to be documented.

1.3.4.5 Smoking: Although smoking is a major factor triggering the pathogenesis of pulmonary manifestations of COPD, the contribution of smoking *per se* to skeletal muscle dysfunction in these patients remains unclear. Montes de Oca *et al*. (240) concluded that smoking may trigger quadriceps atrophy, reduction of constitutive NOS production, and increased muscle fibre glycolytic activities, yet play no role in fibre-type distribution changes, inflammatory cell infiltration, oxidative stress, or reduction in capillary density, characteristics that are frequently observed in limb muscles of COPD patients. Further studies are clearly needed to elucidate the functional importance of smoking in COPD-linked pathogenesis of skeletal muscle dysfunction.

1.3.4.6 Oxidative stress: Normal skeletal muscle fibres produce relatively low levels of ROS and RNS species. Under resting conditions and during mild exercise, ROS are generated inside muscle fibres, mainly by mitochondrial oxidative phosphorylation complexes, and appear to positively influence muscle contractility (279). However, excessive production of ROS under certain conditions, coupled with reduced ability of endogenous antioxidants to neutralize ROS, results in the development of a state of oxidative stress that triggers several biochemical alterations that can be used as indirect markers of oxidative stress. These alterations include peroxidation of membrane phospholipids and fragmentation of fatty acids, both of

which result in production of highly reactive aldehydes such as malonaldehyde (MDA) and 4-hydroxy-2-nonenal (HNE) (129). Oxidative stress is also associated with the formation of carbonyl groups that are generated through selective oxidation of arginine, lysine, threonine, and proline (67). Increased levels of lipid peroxidation by-products and accumulation of carbonyl groups have been documented in skeletal muscles of COPD patients (20, 21, 23). In addition, the observation that exercise induces significantly greater ROS production in limb muscles of COPD patients suggests that they have a higher capacity for oxidative stress induction in response to repeated activation (274). Direct evidence of increased ROS production in resting limb muscles of COPD patients has also been published (28). Increased RNS generation, as indicated by protein tyrosine nitration, has also been documented in the diaphragms of COPD patients (19).

There are many sites inside skeletal muscles of COPD patients that are involved in increased ROS production. Mitochondria are a major source of ROS production as a result of electron leaks from the oxidative phosphorylation chain of protein complexes located on the inner membrane of mitochondria. Picard *et al.* (28) have recently documented that mitochondrial H_2O_2 release in isolated muscle strips from quadriceps biopsies of COPD patients is significantly greater than in control subjects. One likely explanation for this finding is the fact COPD patient muscles have greater proportions of type II fibres, which exhibit lower ROS scavenging than do type I fibres (293).

Another possible source of enhanced ROS generation is xanthine oxidase, which is derived from xanthine dehydrogenase, an enzyme responsible for oxidation of xanthine and hypoxanthine. However, there is little information available

regarding the importance of xanthine oxidase to oxidative stress in limb muscles of COPD patients. Heunk *et al*. (141) have reported that in exercising COPD patients, oxidized glutathione plasma levels and lipid peroxide by-products were significantly attenuated when patients were pre-treated with the xanthine oxidase inhibitor allopurinol. It should be emphasized that oxidative stress in skeletal muscles of COPD patients may also be the result of reduction in antioxidant capacity, particularly the non-enzymatic type. This notion is supported by observations that plasma levels of non-enzymatic antioxidants are lower in COPD patients, both at rest and after localized exercise, than they are in control subjects (62, 275). In addition, muscle enzymatic antioxidant responses are blunted when faced with increased ROS generation in COPD patients. Indeed, when quadriceps muscle is activated through selective exercise protocols, muscle glutathione peroxidase activity rises significantly in control subjects but not in patients with moderate COPD (63). Similarly, Mn-SOD activity fails to increase in response to endurance training protocols in patients with COPD while significant induction of this activity is observed in muscles of control subjects (24).

Deleterious effects of oxidative stress on skeletal muscle contractile performance are believed to be mediated through selective post-translational modifications of several enzymes involved in mitochondrial respiration, glycolysis, and Ca^{++} flux. Mitochondrial complexes I, II, and IV are possible targets of ROS and RNS (59, 246). Aconitase and creatine kinase activities are also inhibited by ROS (23, 39). In quadriceps muscles of COPD patients, creatine kinase is carbonylated and its activity is inhibited as a result of oxidative modification (23). Other ROS and RNS targets inside skeletal muscles, including several glycolytic enzymes such as enolase,

aldolase, and glyceraldehyde 3-phosphate dehydrogenase, are modified and inhibited by lipid peroxidation products and peroxynitrite (22, 152, 326, 362). Proteins modified by ROS and RNS inside skeletal muscles include ryanodine receptor channels (RyR) (334), $Na^{+/}K^{+}$ pump (198), and Ca^{++} ATPase (185, 363). Finally, ROS and RNS may also target several myofibrillar proteins, including actin, MHC, myosin light chain, tropomyosin, and actinin (169, 175, 234, 362). Their modification leads to increased degradation, alterations in function, and changes in sensitivity to Ca^{++}.

1.3.4.7 Alterations in protein synthesis and degradation: Skeletal muscle mass is regulated by a balance between protein synthesis and protein degradation. The latter is achieved through four different systems: the ubiquitin-proteosomal system (UPS); the autophagy-lysosomal system; the calpain system; and the caspase system. The UPS is dependent on targeted protein tagging by ubiquitin, a process that involves activation of ubiquitin by E1 ubiquitin-activating proteins, a transfer of activated ubiquitin to E2 ubiquitin conjugating enzymes, and, finally, eventual transfer of ubiquitin to targeted proteins by E3 ubiquitin ligases. Skeletal muscles express different types of ubiquitin-activating and ubiquitin-conjugating enzymes but primarily express two E3 ligases known as muscle ring finger (MuRF1) and muscle atrophy F-box (MAFbx or atrogin-1)(34). Their expressions are significantly induced inside muscle fibres in various models of muscle atrophy (34). Upstream signals that mediate the activation of the UPS in skeletal muscle atrophy remain unclear. However, recent studies have indicated that expressions of atrogin-1 and MuRF1 are controlled by a complex signalling network comprised of FoxO transcription factors (294) and their upstream regulators, including AKT (41).

The autophagy-lysosomal system results in the formation of double-membrane vesicles (autophagosomes) that engulf cytosolic proteins as well as organelles such as mitochondria and peroxisomes. The cargo of autophagosomes is delivered to lysosomes, which contain several cathepsins (acid hydrolases), glycosidases, lipases, nucleases, and phosphatases; these are responsible for degradation of the cargo (276).

Calpains consist of fourteen different cysteine proteases that are dependent on Ca^{++}. Skeletal muscles contain substantial levels of two ubiquitous calpains, one that requires micromolar concentrations of Ca^{++} (μ-calpain), and one that requires millimolar levels of Ca^{++} (m-calpain), in addition to the calpain inhibitor calpstatin (26). Calpains cleave proteins at selective sites and are not involved in the degradation of cytosolic proteins inside skeletal muscles.

Caspases are responsible for degrading cellular proteins during apoptosis. Several recent reports have confirmed that caspase activity and/or expression inside skeletal muscle is elevated in muscular dystrophy, sepsis, and exercise-induced oxidative stress (149, 189, 335).

Many investigators believe that limb muscle atrophy in patients with COPD is mediated by an imbalance between protein synthesis and degradation (76). No information is as yet available regarding changes in skeletal muscle protein synthesis in COPD patients. Little is known about exact contributions of each proteolysis program to overall protein degradation in skeletal muscles of COPD patients. There is evidence, however, that the ratio of catabolic factors, such as cortisol and IL6, to anabolic factors, such as insulin-like growth factor 1 (IGF1), is significantly elevated

in limb muscles of COPD patients (78). These changes suggest that there is an imbalance in protein synthesis/degradation. In addition, expressions of FoxO1 and FoxO3a transcription factors, which are major transcriptional regulators of MuRF1 and atrogin-1 E3 ligases, have recently been shown to be significantly induced in quadriceps muscles of COPD patients, suggesting that the UPS system is activated to higher levels than it is in control subjects (77, 83). It should be noted that both FoxO1 and FoxO3 transcription factors can induce muscle atrophy by inhibiting IGF1-medited protein synthesis (331).

Upregulation of the activity of UPS in COPD patients is not limited to limb muscles. Ottenheijm *et al.* (259) described a more than 3-fold induction of atrogin-1 in the diaphragm of patients with moderate COPD, suggesting that even respiratory muscles may undergo upregulation of protein degradation in these patients. The exact mechanisms involved in inductions of atrogin-1, MuRF, and FoxO1 and FoxO3a transcription factors in skeletal muscles of COPD patients remain under investigation, but one possibility is that activation of NFκB transcription factor may be involved. This factor has a strong influence on MuRF1 expression. Overexpression of IKKβ, an upstream regulator of NFκB, in skeletal muscles of normal mice results in significant atrophy and MuRF1 upregulation (41). Evidence of NFκB activation in skeletal muscles of COPD patients was provided by Agusti and colleagues (7), who reported a 30% increase in NFκB DNA binding activity in quadriceps biopsies of patients with COPD and severe muscle wasting.

Despite evidence of increased proteolysis as an important cause of skeletal muscle atrophy and poor contractile performance in COPD patients, no information is

as yet available regarding relative contributions of the autophagy-lysosomal, calpain, and caspase proteolysis systems to the atrophy process in these patients.

1.3.4.8 Reduction in vascular density and capillarization: It has been well established that peak oxygen consumption during exercise in COPD patients is significantly lower than control subjects. Oxygen delivery and blood flow are critical factors in determining skeletal muscle endurance. One of the major determinants of adequate blood flow distribution within skeletal muscle fibres is capillary density and the ratio between number of capillaries and muscle fibres. Very few studies have addressed whether alterations in capillary density develop and contribute to poor exercise performance by skeletal muscles of COPD patients. An initial study by Jobin and colleagues (159) described significant reduction in the number of capillaries per mm^2 in VL muscles of patients with moderate COPD as compared to control subjects. This reduction in capillarity persists even when capillary density is expressed as capillary per fibre ratio. Subsequent studies have confirmed this observation {3990}. Capillary density is regulated by complex interactions between pro-angiogenic factors such as VEGF, FGFs, PDGFs, and angiopoietins, which promote angiogenesis and capillary network stability, and angiostatic factors, which inhibit angiogenesis (33). In patients with moderate and severe COPD, there is no information as to whether reduced limb muscle contractile function (strength and endurance) is mediated in part through abnormalities in angiogenesis factor production. To the best of our knowledge, only three studies have been published that evaluate VEGF family member expression in skeletal muscles of COPD patients. Barreiro *et al.* (25) has reported that VEGF protein levels in VL muscles of patients with moderate and severe COPD are significantly lower than those of control subjects. These observed reductions in

VEGF levels are not likely to have been caused by inactivity or changes in fibre-type distribution within skeletal muscle because VEGF levels didn't correlate with fibre-type distribution or the degree of exercise tolerance in these patients (25). These results contrast with those of Jatta *et al.* (157) who described the absence of any significance differences in mRNA levels of VEGFA, VEGFB and VEGFC genes in TA muscles of patients with severe COPD, as compared to control subjects. VEGF expression in VL muscles of COPD patients contrasts with that in the diaphragm, which exhibits mild, but significant, elevations in VEGF levels in patients with moderate COPD (10). It should be noted that upregulation of VEGF expression in diaphragms of COPD patients, which are not paralleled by increases in FGF1 or TGFβ expression, cannot be attributed to increased mechanical loads in these patients, although other local factors such as fibre-type remodelling and hypoxemia may be involved. The involvement of pro- and anti-angiogenesis factors other than VEGF in the regulation of skeletal muscle capillary density in COPD patients remains to be explored.

It should be emphasized that apart from these three studies, which address changes in VEGF expression, there is no information as yet available regarding other angiogenesis-related factor or inhibitor expression in skeletal muscles of COPD patients. In chapter 3 of this thesis, we have addressed this lack of information regarding changes in pro- and anti-angiogenesis factor expression in limb muscles of COPD patients by performing real-time PCR arrays to evaluate expressions of VEGF, Ang-1 and Ang-2. The Hussain laboratory screen of various angiogenesis-related genes also revealed that Ang-2 (ANGPT2) expression is significantly upregulated in

the VL muscle of COPD patients as compared to control subjects. No information is yet available regarding skeletal muscle production of Ang-2. Influences of Ang-2 on skeletal muscle satellite cell survival, differentiation, and proliferation are also unknown. The main objectives of chapter 3 of this thesis are to understand the mechanisms that underlie the induction of Ang-2 in limb muscles of COPD patients and to explore the functional significance of elevated Ang-2 expression in these muscles.

PREFACE TO CHAPTER 2

Ang-1 is known to exert important effects on the inflammatory responses in endothelial cells. Ang-1 inhibits pro-inflammatory cytokine-induced adhesion molecule expression on the surface of endothelial cells and attenuates vascular leakage both *in-vivo* and *in-vitro* conditions. Moreover, systemic administration of viruses expressing Ang-1 improves survival and attenuates vascular derangements in mice with vascular failure. While Ang-1 antagonizes inflammatory responses in the vasculature, Ang-2 potentiates these responses particularly those elicited by TNFα. Indeed, TNFα-induced leukocyte adhesion to endothelial cells is potentiated by endogenously-released Ang-2.

It is because of these important effects of angiopoietins on inflammatory responses that investigators have long been interested in identifying how alterations in endogenous angiopoietin contribute to the pathogenesis of septic shock, a serious disease in intensive care units where pro-inflammatory cytokines play crucial roles. However, the nature of angiopoietin contribution to the vascular associated with septic shock remains unclear primarily because little is known about the influence of pro-inflammatory mediators on organ-specific regulation of angiopoietin production and Tie-2 receptor expression.

In chapter 2, I used a murine model of septic shock induced by bacterial lipopolysaccharide (LPS) as well as cultured epithelial and skeletal muscle cells to address how septic shock induced by LPS administration alters endogenous angiopoietin expression. My hypothesis was that exposure to LPS elicits significant upregulation of Ang-2 while inhibiting simultaneously the expression of Ang-1 and

Tie-2 receptors. I also hypothesized that the transcription factor NFκB promotes the induction of Ang-2 expression in response to LPS exposure but not the inhibition of Ang-1 expression.

CHAPTER 2

REGULATION OF ANGIOPOIETIN EXPRESSION BY BACTERIAL LIPOPOLYSACCHARIDE

REGULATION OF ANGIOPOIETIN EXPRESSION BY BACTERIAL LIPOPOLYSACCHARIDE

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2.1 ABSTRACT

Angiopoietins are ligands for Tie-2 receptors and play important roles in angiogenesis and inflammation. While angiopoietin-1 (Ang-1) inhibits inflammatory responses, angiopoietin-2 (Ang-2) promotes cytokine production and vascular leakage. In this study, we evaluated in vivo and in vitro effects of *E. coli* lipopolysaccharides (LPS) on angiopoietin expression. Wild type C57/BL6 mice were injected with saline (control) or *E. coli* LPS (20 mg/ml) i.p. and sacrificed 6, 12 and 24 hrs later. The diaphragm, lung and liver were excised and assayed for mRNA and protein expression of Ang-1, Ang-2, and Tie-2 protein and tyrosine phosphorylation. LPS injection elicited several fold rise in Ang-2 mRNA and protein levels in the three organs. By comparison, both Ang-1 and Tie-2 levels in diaphragm, liver and lung were significantly attenuated by LPS administration. In addition, Tie-2 tyrosine phosphorylation in the lung was significantly reduced in response to LPS injection. *In vitro* exposure to *E. coli* LPS elicited a cell-specific changes in Ang-1 expression with significant induction in Ang-1 expression being observed in cultured human epithelial cells, whereas significant attenuation of Ang-1 expression was observed in response to *E. coli* LPS exposure in primary human skeletal myoblasts. In both cell types, *E. coli* LPS elicited substantial induction of Ang-2 mRNA, a response that was mediated in part through NFκB. We conclude that *in vivo* endotoxemia triggers functional inhibition of the Ang-1/Tie-2 receptor pathway by reducing Ang-1 and Tie-2 expression and inducing Ang-2 levels and that this response may contribute to enhanced vascular leakage in sepsis.

Key words: Sepsis, Lipopolysaccharide, inflammation, angiopoietins, NFkB.

2.2 INTRODUCTION

Angiopoietin-1,-2,-3 and -4 are oligomeric-secreted glycoproteins that are ligands for endothelial cell-specific Tie-2 receptors (4). Angipoietin-1 (Ang-1) is released by fibroblasts, vascular smooth muscles, thyrocytes, tumour cells and endothelial (EC) cells, whereas angiopoietin-2 (Ang-2) is produced primarily by ECs (4). Angiopoietin-3 (Ang-3) and its human ortholog, Ang-4, are more recently identified members of the angiopoietin family, with Ang-3 having wide tissue distribution in mice, and Ang-4 being most strongly expressed in human lungs (33). Ang-1 and Ang-2 bind Tie-2 receptors with equal affinity, although Ang-2 elicits only context-sensitive phosphorylation of these receptors and can competitively inhibit Ang-1-induced phosphorylation of Tie-2 (21).

In addition to its crucial roles in embryonic vascular development (29), the Ang-1/Tie-2 receptor pathway promotes EC migration, proliferation, survival and differentiation (28). Moreover, Ang-1 protects adult peripheral vasculature from vascular leakage (31) and inhibits the effects of pro-inflammatory cytokines on ECs. Indeed, when Ang-1 is co-incubated with vascular endothelial growth factor (VEGF) or tumor necrosis factor (TNFα), leukocyte adhesion to ECs, migration of leukocytes across ECs, and the expression of adhesion molecules are all strongly inhibited (16,17). Unlike Ang-1, the effect of Ang-2 on angiogenesis is more complex and is context dependent; when Ang-2 levels are elevated, enhanced angiogenesis is apparent when VEGF is present, whereas vascular regression has been observed in its absence (11).

There is increasing evidence that Ang-2 may promote EC vascular leakage both *in-vitro* and *in-vivo* (24,26). Ang-2 also promotes adhesion of leukocytes to ECs by sensitizing the latter towards TNF-α and increases expression of adhesion molecules on the surface of ECs (6). The biological roles of Ang-3 and Ang-4 remain unclear. Although initial reports suggest that Ang-4 stimulates Tie-2 phosphorylation in a fashion similar to that elicited by Ang-1, and that Ang-3 does not activate Tie-2 receptors (33), later studies have confirmed that both Ang-3 and Ang-4 promote EC survival, proliferation and differentiation (14).

Septic shock is caused by an exaggerated systemic inflammatory response to gram-negative bacteria and the cell wall component, lipopolysaccharide (LPS). In the United States, severe sepsis and septic shock are major causes of mortality in intensive care units and account for more than 750,000 cases per year, with an estimated mortality of ~30% (2). Septic shock is characterized by increased capillary permeability, widespread EC dysfunction, alveolar and interstitial pulmonary edema and increased expression of pro-inflammatory cytokines and chemokines, including TNF-α, IL-1, IL-6 and interferon-γ. The involvement of angiopoietins in the pathogenesis of septic shock is as yet unclear, primarily because little information is available regarding *in-vivo* regulation of angiopoietin production in various organs during sepsis or septic shock. *In-vitro* studies have revealed opposing effects of pro-inflammatory cytokines such as TNFα, interleukin-1β (IL-1β) and interferon γ (IFNγ) on Ang-1 gene expression. While TNFα and IL-1β stimulate Ang-1 expression in synovial fibroblasts and ECs (5,9), a combination of TNFα and IFNγ reduces Ang-1 levels in cultured osteoblasts (13). As for Ang-2 gene expression, it has been shown

that pro-inflammatory stimuli strongly activate Ang-2 transcription in ECs (9,15,22) and two recent studies have documented elevated circulating levels of Ang-2 protein in patients with sepsis (23,24).

Despite the importance of bacterial lipopolysaccharides (LPS) in the pathogenesis of sepsis and septic shock, little is known about the influence of LPS on the regulation of angiopoietin expression. Brown *et al.* (5) have reported that *E. coli* LPS induced Ang-1 mRNA expression and promoter activity in cultured fibroblasts. In contrast, Karmpaliotis *et al.* (12) have studied the *in-vivo* regulation of angiopoietins in a murine model of LPS-induced acute lung injury and have reported a significant decline in epithelial Ang-1 and Ang-4 mRNA and protein expressions 96 hrs after administration of nebulized LPS (12). These authors have also found that in response to LPS administration Ang-2 expression in airway epithelial cells declines significantly, whereas that of alveolar cells increases (12). These studies leave many important questions regarding the influence of LPS on angiopoietin expression unanswered. In this study, we used a murine model of LPS-induced sepsis and cultured cell models to address the influence of *in vivo* and *in vitro* effects of LPS on angiopoietin expression and to evaluate the role of NFκB in this regulation. Our hypothesis was that LPS administration would elicit downregulation of Ang-1 expression while simultaneously inducing Ang-2 expression, with the overall response being inhibition of the Ang-1/Tie-2 receptor pathway. We also hypothesized, on the basis of previous studies on Ang-1 and Ang-2 promoters (5,10,22) that NFκB plays an important role in LPS-induced Ang-2 expression, but not in the downregulation of Ang-1 expression brought upon by LPS administration.

2.3 METHODS

2.3.1 ANIMAL PREPARATION

Adult (8-12 week old) male wild type C57/Bl6 (n=36) mice were studied at the McGill University Animal Facility and were divided into two major groups. Animals in group 1 were injected i.p. with saline and sacrificed 6, 12 and 24 hrs later (n=6 at each time point). Animals in group 2 received i.p. injection of *E. coli* LPS (20 mg/kg, serotype 055:B5, Sigma-Aldrich Co. (Oakville, ON)) and were sacrificed 6, 12 and 24 hrs later (n=6 at each time point). All animals were euthanized with pentobarbital sodium followed by quick excision of the liver, diaphragm, and lungs, which were flash-frozen in liquid nitrogen and stored at -80°C until further analysis.

2.3.2 CELL CULTURE

It has been well established that systemic exposure to LPS initiates a rapid, coordinated recruitment of inflammatory cells and overproduction of pro-inflammatory mediators which might indirectly regulate tissue angiopoietin expression in response to *in vivo* LPS administration. To study the primary regulation of angiopoietin gene expression by LPS signaling, we evaluated angiopoietin gene expression in cultured epithelial cells (representative of pulmonary cells) and skeletal muscle myoblasts (representative of skeletal muscles) exposed to *E. coli* LPS for up to 24 hrs. We have verified in pilot experiments that these two cell types produce mainly Ang-1 and have no detectable Tie-2 receptor expression.

Epithelial cell culture: The adeno12 SV40-transformed human bronchial epithelial cell line, BEAS-2B, was a gift from Dr. C. Harris (National Cancer Institute, Bethesda, MD) and was cultured in DMEM/F12 (Invitrogen Inc., Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-

glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). BEAS-2B cells were exposed to either phosphate buffered saline (PBS, control) or 0.1 μg/ml of *E. coli* LPS (serotype 055:B5, Sigma-Aldrich Co.) for 6, 12 and 24 hrs. To evaluate gene expression of ESE-1 transcription factor, cells were exposed to PBS or LPS for 3, 6 and 12 hrs. Cells were then harvested and total RNA was extracted (see below). We verified in preliminary experiments that this concentration of LPS elicits a substantial induction of interleukin-8 protein production in the culture medium as measured by ELISA (R&D Systems, Minneapolis, MN). To evaluate the involvement of the NFκB pathway in LPS-induced regulation of angiopoietin expression in epithelial cells, we used adenoviruses expressing GFP (Ad-GFP) or a dominant negative mutant form (K44A) of IκB kinase β (IKKβ) (Ad-dnIKKβ) (27). BEAS-2B cells (60-70% confluent) were infected overnight at a multiplicity of infection (moi) of 100 in serum-free medium. The virus-containing medium was then replaced with complete medium containing 5% FBS and incubated for 24 hrs. Cells were then exposed for 6 hrs to either PBS (control) or *E. coli* LPS, as described above.

Human skeletal muscle myoblasts: Primary human skeletal muscle myoblasts, immortalized by expression of the E6E7 early region of human papillomavirus type 16, were a kind gift from Dr. E. Shoubridge (McGill University, Montréal, QC). Myoblasts were cultured in SkBM using the SkBM® Bulletkit® (Cambrex Research Products, Walkersville, MD) supplemented with 15% inactivated FBS. Sub-confluent myoblasts were exposed either to PBS (control) or 10 μg/ml of *E. coli* LPS (serotype 055:B5, Sigma-Aldrich Co.) for 6, 12 and 24 hrs. To evaluate gene expression of ESE-1 transcription factor, cells were exposed to PBS or LPS for 3, 6 and 12 hrs.

Cells were then lysed and total RNA was extracted (see below). To evaluate the involvement of NFκB transcription factor in LPS-induced changes in angiopoietin expression in myoblasts, cells were infected overnight (1000 moi) with adenoviruses expressing GFP and a dominant-negative form of IKKβ, as described above. The virus-containing medium was then replaced with complete medium containing 15% FBS, cells were incubated for 24 hrs and then exposed for 6 hrs to either PBS (control) or *E. coli* LPS, as described above.

2.3.3 RNA EXTRACTION AND REAL TIME PCR

Total RNA was extracted from mouse liver, diaphragm, and lung samples, as well as from cultured cells using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Co.). Quantification and purity of total RNA was assessed by A_{260}/A_{280} absorption. Total RNA (2 μg) was then reverse transcribed using Superscript II® Reverse Transcriptase kits and random primers (Invitrogen). Reactions were incubated at 42°C for 50 min and at 90°C for 5 min. Real-time PCR was performed using a Prism 7000 Sequence Detection System from Applied Biosystems (Foster City, CA). To quantify the expressions of 18S (endogenous control), murine Ang-1, Ang-2, Ang-3, and VEGF, as well as human Ang-1, Ang-2 and Ang-4 transcripts, we used Applied Biosystems TaqMan® gene expression assays (catalogue# 4352930E, Mm00456503_m1, Mm00545822_m1, Mm00507766_m1, Mm00437304_m1, Hs00181613_m1, Hs00169867_m1, and Hs00211115_m1, respectively). For ESE-1 (ELF3) transcription factor, we used TaqMan® expression assay catalogue# Hs00963882_g1. The thermal profile was as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles (95°C for 15 sec and 60°C

for 1 min). Each PCR reaction was carried out in triplicate on one plate and the results presented are combined from each treatment group. Dissociation curve analyses were performed to show the specificity of amplification. Results were analyzed in two ways. First, we used the comparative threshold cycle (C_T) method to calculate fold changes in expression in the LPS groups compared with the saline groups (20), where:

$\Delta C_T = C_T$ of gene of interest - C_T of 18S

$\Delta\Delta C_T = \Delta C_T$ of LPS-treated animal groups - ΔC_T of saline-treated animal groups

Fold changes in gene expression in the LPS groups were then calculated as $2^{-\Delta\Delta C_T}$. All real-time PCR experiments were performed in triplicate. A similar approach was used to calculate fold changes in expression in LPS-treated cells compared with control cells. Second, to determine the absolute copy numbers of Ang-1, Ang-2 and Ang-3 mRNA transcripts in tissue or cell samples, we established standard curves relating the C_T values of these genes to the copy numbers. These curves were generated by performing Real-Time PCR analysis using angiopoietin TaqMan® primers on samples with known copy numbers of plasmids containing full coding sequences of murine and human Ang-1, Ang-2, Ang-3 and Ang-4 cDNA. Plasmids were diluted serially to generate copy numbers ranging from 30 to 300,000. Copy numbers of angiopoietins were then calculated using these curves and were normalized per 1 ng total RNA obtained from tissue and cell samples.

2.3.4 IMMUNOBLOTTING

Frozen liver, diaphragm, and lung tissues were homogenized in 6 volumes (wt/vol) of homogenization buffer (pH 7.4, 10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml of phenylmethylsulfonyl fluoride, 0.32 mM sucrose, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A). Crude homogenates were centrifuged at 4°C for 15 min at 5,000 rpm. Supernatants were then collected and used for immunoblotting. Tissue proteins (100 μg) were heated for 5 min at 90°C and then loaded onto tris-glycine SDS-polyacrylamide gels, electrophoretically separated, transferred to polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk, and subsequently incubated overnight at 4°C with primary monoclonal antibodies to Ang-1 and Ang-2 (R&D Systems) and a polyclonal anti-Tie-2 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Preliminary experiments indicated that commercially available anti-Ang-3 antibodies are not selective to this protein. Specific proteins were then detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence reagents provided with an ECL kit from Amersham Canada (Oakville, ON). Loading of equal amounts of proteins was confirmed by stripping the membranes and re-probing with anti-α-tubulin antibody (Sigma-Aldrich Co.). The blots were scanned with an imaging densitometer and protein band OD was quantified with Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD). Predetermined molecular mass standards were used as markers.

2.3.5 IMMUNOPRECIPITATION

To investigate whether LPS administration alters Tie-2 receptor tyrosine phosphorylation, we immunoprecipitated Tie-2 receptors and probed them with anti-

phosphotyrosine antibodies. Tissue homogenates (500 mg) were incubated with primary polyclonal anti-Tie-2 receptor antibody for 12 hrs at 4°C. Protein A-agarose conjugates were then added and samples were incubated for a further 3 hrs. After centrifugation, pellets were washed three times with a buffer containing 125 mM Tris-HCl, pH 8.1, 500 mM NaCl, 0.5% Triton-X-100, 10 mM EDTA and 0.02% NaN_3. The final wash was performed with water. Proteins were eluted with electrophoresis sample buffer and immunoblotting of supernatant and eluted proteins was undertaken as described above. Membranes were probed with monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY). Proper negative controls included omission of primary antibody and omission of protein A-agarose conjugates.

2.3.6 DATA ANALYSIS

Data are presented as means ±SD. Six separate animals were studied in each group for each time point and three different organs (liver, diaphragm, and lung) were sampled in each animal. At a given time point, differences between control (saline-injected) and LPS-injected animals in terms of angiopoietin and VEGF expressions were compared using Two-Way Analysis of Variance and P values less than 0.05 were considered significant. Similar analysis in terms of angiopoietin expression was used to compare control (PBS-treated) and LPS-treated epithelial cells and skeletal muscle myoblasts.

2.4 RESULTS

2.4.1 INFLUENCE OF *IN-VIVO* LPS INJECTION

Figure 1 illustrates the influence of *in-vivo* LPS injection on angiopoietin and VEGF mRNA expression in the liver, diaphragm and lung. Expression of Ang-1 mRNA increased significantly after 6 hrs of LPS injection only in the diaphragm. After 12 and 24 hrs of LPS administration, Ang-1 mRNA declined significantly in the liver and diaphragm and after 12 hrs in the lung (Figure 1). Similarly, Ang-3 mRNA in the diaphragm and lung declined after 6, 12 and 24 hrs and in the liver after 6 and 12 hrs of LPS administration. The time course of VEGF mRNA expression in the diaphragm was very similar to that of Ang-1 with an initial increase and a subsequent decline after 12 and 24 hrs of LPS administration (Figure 1). Expression of VEGF in the liver and lung remained unchanged in response to LPS administration. In comparison, Ang-2 mRNA increased substantially in the three organs through the time course of LPS injection (Figure 1). To validate whether these changes in mRNA expression were associated with similar changes in protein levels, we performed immunoblotting for Ang-1 and Ang-2 proteins using selective antibodies. Figure 2 shows that injection of LPS after 12 and 24 hrs resulted in a significant decline in Ang-1 and an increase in Ang-2 protein levels in the liver, diaphragm and lung.

Liver, diaphragm, and lung Tie-2 protein levels declined significantly in response to LPS administration (Figure 3). We were only able to detect Tie-2 tyrosine phosphorylation in lung samples. This could be due to a relatively higher abundance of Tie-2 expression in the lung as compared to other tissues. LPS administration resulted in a significant decline in Tie-2 tyrosine phosphorylation (Figure 3C). This reduction is not simply due to a reduction in Tie-2 protein levels since the ratio of

tyrosine phosphorylated Tie-2 to total Tie-2 declined significantly in the lungs of LPS-injected animals ($P<0.05$ compared with saline-injected animals).

2.4.2 EFFECTS OF *IN-VITRO* LPS EXPOSURE

Table 1 lists the abundance of Ang-1, -2 and -4 mRNA transcripts in BEAS-2B epithelial cells and primary human skeletal myoblasts. BEAS-2B cells express relatively lower levels of these transcripts as compared with primary skeletal myoblasts (Table 1). Exposure of BEAS-2B cells to LPS elicited a significant induction in Ang-1 and Ang-2 mRNA expression, which peaked after 12 hrs of LPS administration (Figure 4A). In comparison, Ang-4 mRNA expression declined significantly after 12 and 24 hrs of LPS exposure (Figure 4). Inhibition of the NFκB transcription factor activation using a dominant-negative form of IKKβ had no influence on the 12 hr-response of Ang-1 and Ang-4 transcripts to LPS administration; however, it significantly attenuated LPS-induced induction of Ang-2 mRNA (Figure 4B).

Primary human skeletal myoblasts express relatively higher levels of Ang-1 mRNA transcripts compared to those of Ang-2 and Ang-4 (Table 1). LPS exposure of these cells significantly attenuated Ang-1 and Ang-4 mRNA expressions while Ang-2 mRNA was significantly induced by LPS in these cells (Figure 5). Expression of a dominant-negative form of IKKβ had no influence on the decline in Ang-1 and Ang-4 expression, but it completely eliminated LPS-induced Ang-2 expression (Figure 5B).

Figure 6A illustrates the time course of mRNA expression of ESE-1 (ELF3) transcription factor in both BEAS-2B epithelial cells and skeletal myoblasts in response to PBS (control) and LPS exposure. ESE-1 expression rose substantially

after 3 hrs of LPS exposure with a decline in the expression thereafter. After 12 hrs of LPS exposure, ESE-1 levels measured in response to LPS exposure in both cells types were not different from those measured in the presence of PBS. Infection of epithelial and skeletal myoblast cells with adenoviruses expressing GFP did not alter the induction of ESE-1 mRNA in response to 3 hrs exposure to LPS (Figure 6B). In comparison, expression of a dominant negative form of IKKβ completely blocked the effect of LPS exposure on ESE-1 mRNA expression (Figure 6B). These results indicate that LPS-induced ESE-1 mRNA expression in both cell types is dependent on NFκB activation.

2.5 DISCUSSION

The main findings of this study include: 1) *In vivo* LPS administration elicits differential effects on angiopoietin and Tie-2 receptor expressions. While expressions of Ang-1, Ang-3 and Tie-2 receptors decline significantly in the liver, diaphragm and lung, Ang-2 mRNA expression in these organs is significantly induced. 2) *In vitro* LPS exposure elicits cell-specific changes in Ang-1 expression. Ang-1 mRNA is induced in BEAS-2B epithelial cells but is significantly attenuated in skeletal myoblasts. In comparison, LPS induces Ang-2 mRNA levels and attenuates Ang-4 expression in both cell types. While the induction of Ang-2 expression in both cell types is mediated in part through NFκB, this transcription factor had no direct role in the influence of LPS on Ang-1 and Ang-4 expressions in either cell type. 3) LPS elicits a significant induction of ESE-1 mRNA expression in epithelial cells and skeletal myoblasts, a response which is dependent on NFκB activation.

In addition to their roles in the regulation of angiogenesis, there is increasing evidence that angiopoietins regulate inflammation, with Ang-1 exerting anti-inflammatory effects by inhibiting leukocyte-endothelial cell adhesion and transmigration, reducing inflammatory mediator-induced vascular leakage and cytokine production, and by attenuating EC adhesion molecule expression (17,16,25,7). Moreover, overexpression of Ang-1 improves survival and homodynamic functions, reduces lung injury, and attenuates the expression of adhesion molecules in mice with LPS-induced acute lung injury (32). In contrast, upregulation of Ang-2 production by ECs exposed to pro-inflammatory mediators such as TNFα, thrombin and angiotensin II suggest that Ang-2 may promote

inflammation (22,15). This suggestion is supported by findings of elevated circulating Ang-2 levels in patients with severe sepsis and in children with septic shock, and by the observation that injection of Ang-2 protein *in vivo* elicits a significant increase in edema formation in the mouse paw (26,8,23). Finally, Ang-2 deficient mice have an impaired ability to express cytokine-inducible adhesion molecules on EC surfaces after inflammatory activation, further implicating Ang-2 in promoting inflammation (6). No information is as yet available as to whether Ang-3 and Ang-4 regulate inflammation; however, the observations that both Ang-3 and Ang-4 activate Tie-2 receptors suggest that these angiopoietins may have similar anti-inflammatory effects to those of Ang-1 (19).

Despite increasing evidence of important roles for angiopoietins in regulating inflammation, little is known about how pro-inflammatory stimuli such as LPS influence endogenous angiopoietin production. Thus far, two studies have addressed the influence of LPS on Ang-1 expression, but have resulted in contradictory conclusions. Brown *et al*. (5) reported a significant induction of Ang-1 mRNA expression by LPS in fibroblasts. In comparison, *in vivo* LPS administration into murine lungs was reported to attenuate pulmonary epithelial Ang-1 expression, a response that was associated with increased vascular leakage and infiltration of lung interstitia by inflammatory cells (12). The present study is the first to describe both the *in vivo* and *in vitro* effects of LPS on the expression of the four angiopoietins in various organs and cells. We report here that *in vivo* administration of LPS in mice is associated with attenuation of Ang-1 and Ang-3 expressions in at least three organs- liver, diaphragm and lung - while Ang-2 expression in these organs increases significantly (Figure 1). These changes in angiopoietin expression are associated with

downregulation of Tie-2 receptor expression and reduction in Tie-2 tyrosine phosphorylation. We conclude, on the basis of these findings, that *in vivo* LPS administration causes functional inhibition of the angiopoietin/Tie-2 receptor pathway in at least these three organs and that this inhibition is mediated by three mechanisms, namely, reduced expression of Tie-2 receptor agonists (Ang-1 and Ang-3), induction of Ang-1 antagonist (Ang-2) and reduction in Tie-2 receptor expression. Although we did not directly assess the implications of an elevation of Ang-2 expression and downregulation of Ang-1, Ang-3 and Tie-2, we speculate that these alterations likely contribute to hemodynamic derangements and enhancements of inflammatory responses in sepsis since attenuation of Ang-1 and increased Ang-2 expression are likely to result in enhanced vascular leakage and augmented expression of pro-inflammatory mediators and adhesion molecules on the surface of ECs.

It should be pointed out that the decline in pulmonary Ang-1 and Ang-3 expression found in our study is qualitatively similar to that observed by Karmpaliotis *et al.* (12) in mice exposed to inhaled LPS. However, unlike the significant induction of lung Ang-2 expression observed in our study, these authors failed to detect changes in pulmonary Ang-2 expression in their study. We attribute these variances in pulmonary Ang-2 expression to methodological differences in terms of the routes of LPS administration. Ang-2 originates primarily from endothelial cells and pulmonary endothelial cells are likely to be the largest contributor to total Ang-2 mRNA expression measured in lung samples. Pulmonary endothelial cells and Ang-2 promoter activity are likely to be strongly activated by circulating LPS administered systemically as in our study compared with inhaled LPS administration where LPS

are likely to be deposited mainly on airway and pulmonary epithelial cells. Another issue that could explain the differences in Ang-2 regulation is that we studied Ang-2 expression during early phases of endotoxemia (6 to 24hrs) whereas Karmpaliotis *et al.* studied the late phases of LPS-induced acute lung injury (24 to 96 hrs). It is possible that LPS-induced upregulation of Ang-2 expression is a transient phenomenon and might not be detected during the late phases of endotoxemia.

The results shown in Figure 1 reveal three new aspects of *in vivo* regulation of angiopoietins in sepsis. First, the expression of both Ang-1 and Ang-3 mRNA in the three organs declines after 12 and 24 hrs of *in vivo* LPS administration, suggesting the existence of common mechanisms involved in regulating these two angiopoietins in response to LPS administration. The nature of these mechanisms remains to be elucidated. We should point out that the similarity in the time course of Ang-1 and Ang-3 expression in mice injected with LPS has never before been reported. In fact, opposite regulation of these changes has been observed in the lungs of hypoxic rats, where Ang-3 expression is significantly induced while that of Ang-1 expression is attenuated (1). Second, the fact that LPS injection elicits a significant upregulation of Ang-2 mRNA and protein in at least three organs implies that increased circulating levels of this protein in patients with severe sepsis may originate from more than one organ (8,23). Thirdly, there is qualitative similarity in the responses of Ang-1 and VEGF expressions in the diaphragm to *in vivo* LPS administration, with the expression of both genes being induced within 6 hrs, followed by significant attenuation thereafter. These results suggest that previously reported upregulation of VEGF expression in septic animals is only a transient response (3) and that prolonged effects of LPS include the inhibition of VEGF expression. The similarity in the time

course of Ang-1 and VEGF expression in the diaphragm during the course of endotoxemia also suggests that common pathways are involved in regulating these two pro-angiogenesis factors in the diaphragm.

The exact mechanisms through which *in vivo* LPS exposure elicits a decline in Ang-1 and Ang-3 expression and the induction of Ang-2 expression remain unclear. To investigate the involvement of the NFκB transcription factor in these processes, we used *in vitro* cultured epithelial cells and skeletal myoblasts and infected these cells with adenoviruses expressing a dominant negative form of IKKβ. This construct has been shown to inhibit LPS-induced NFκB-mediated signaling in macrophages (27). Our results indicate that in both cell types, LPS-induced elevation of Ang-2 mRNA is completely eliminated by expression of dominant-negative IKKβ, whereas LPS-induced changes in Ang-1 and Ang-4 levels are not altered by this intervention (Figures 4 and 5).

Molecular cloning of human Ang-2 promoter has revealed putative binding sites for the Ets family of transcription factors and also for GATA factors, c-Rel, Smad3, Smad4, AP1-1, AP-2 and Sp1 in a fragment spanning 650 bp around the transcription start site (-109 to +476)(10). This fragment was also identified to be sufficient to control endothelial-cell-specific cytokine induction of Ang-2. The involvement of NFκB transcription factor in the regulation of Ang-2 expression is not clearly understood because thus far analyses of human Ang-2 promoter have not revealed abundant putative sites for NFκB binding. It is possible; however, that NFκB transcription factor is involved indirectly in the activation of Ang-2 promoter through cooperative interactions with other transcription factors, particularly Ets

proteins. Indeed, both NFκB and Ets-1 (a strong inducer of Ang-2 expression) cooperate in inducing inducible nitric oxide synthase (iNOS) in embryonic ventricular myocytes exposed to LPS (30).

The failure of a dominant-negative form of IKKβ to inhibit LPS-induced changes in Ang-1 expression suggests that NFκB transcription factor is not a major regulator of Ang-1 expression during *in vitro* LPS exposure. Brown *et al.* (5) have recently concluded that NFκB may not directly bind to Ang-1 promoter but that it could induce the expression of Ang-1 indirectly through the induction of ESE-1 transcription factor, which binds directly to Ang-1 promoter in fibroblasts exposed to LPS, TNFα and IL1β. To evaluate the involvement of ESE-1 in LPS-induced changes in Ang-1 expression, we measured the time course of ESE-1 mRNA expression in cultured epithelial cells and skeletal myoblasts. In both cell types, LPS exposure elicited a strong, albeit transient, induction of ESE-1 mRNA expression (Figure 6). In addition, ESE-1 induction by LPS was strongly inhibited in cells expressing a dominant-negative form of IKKβ, thereby confirming the involvement of NFκB in the regulation of ESE-1 expression. This observation, along with the fact that expression of a dominant negative IKKβ had no effect on LPS-induced alterations in Ang-1 expression in epithelial cells and skeletal myoblasts, suggests that, in these cell types, ESE-1 is not responsible for the regulation of Ang-1 and Ang-4 expression in response to *in vitro* LPS exposure. Clearly, additional studies are required to elucidate the mechanisms through which LPS regulates Ang-1 and Ang-4 (Ang-3) expression both *in vivo* and *in vitro* conditions.

It should be noted that while LPS exposure triggered similar changes in Ang-1 expression in skeletal myoblasts and the diaphragms of LPS-injected animals, regulation of Ang-1 expression by LPS in cultured epithelial cells was qualitatively different from that observed in the lungs of LPS-injected mice (Figures 1 and 4). The reasons behind these differences are not clear. One possibility is that LPS-induced Ang-1 expression by LPS in cultured epithelial cells is the outcome of primary signaling events triggered by direct activation of TLR-4 receptors whereas secondary responses elicited by mediators such as cytokines, chemokines, growth factors and thrombotic agents released by parenchymal cells and inflammatory cells infiltrating the lungs might have been responsible for reduction in pulmonary Ang-1 expression in mice (Figure 1). Recent studies suggest that two cytokines whose expressions are usually induced by LPS administration may inhibit Ang-1 expression. Indeed, a combination of TNFα and interferon-γ triggered a significant inhibition of Ang-1 expression in osteoblasts and that this effect was mediated by induction of the inducible nitric oxide synthase and enhanced nitric oxide production (13). It is possible that these two cytokines and the resulting increase in pulmonary NO production which usually peaks after 12 hrs of LPS administration in mice (18) could have been involved in the reduction of pulmonary Ang-1 expression in mice. Additional experiments are clearly required to explore the influence of these mediators on the expression of angiopoietins in lung cells.

In summary, our results indicate that LPS administration elicits differential changes in angiopoietin expression both *in vivo* and *in vitro* settings and that the overall response to LPS administration is a functional inhibition of the Ang-1/Tie-2

receptor pathway. We speculate, on the basis of these results, that inhibition of this pathway may have deleterious effects on vascular function in the settings of sepsis and septic shock.

2.6 ACKNOWLEDGMENTS

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2.8 TABLE 1

mRNA expression (expressed as copies per 1000 copies of 18S) of angiopoietins measured as baseline values in the livers, diaphragms and lungs of wild type C57/Bl6 mice and in BEAS-2B epithelial cells and human skeletal myoblasts.

Organ	**Ang-1**	**Ang-2**	**Ang-3**	**Ang-4**
Liver	12±0.7	23±5*	1.3±0.5*	
Diaphragm	1447±92	203±34*	28±12*	
Lung	446±80	280±98*	26±10*	
BEAS-2B cells	0.023±0.0015	0.091±0.063*		0.003±0.002*
Skeletal myoblasts	109±10	9.3±2.2*		0.06±0.011*

Values are means ±SD (n=6 for mouse tissues and n=15 for cells). *P<0.05 compared with Ang-1.

2.9 FIGURES

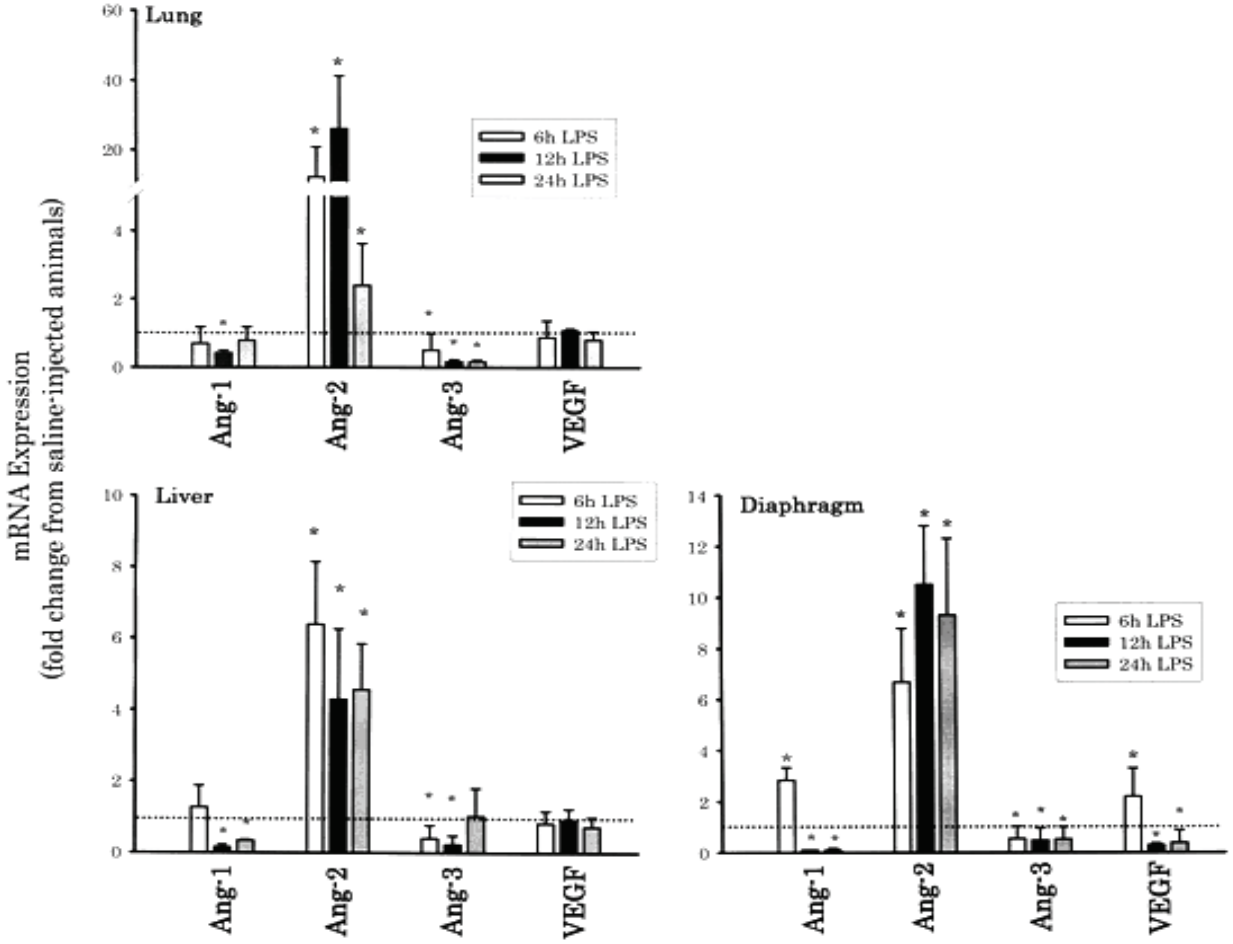


Figure 1

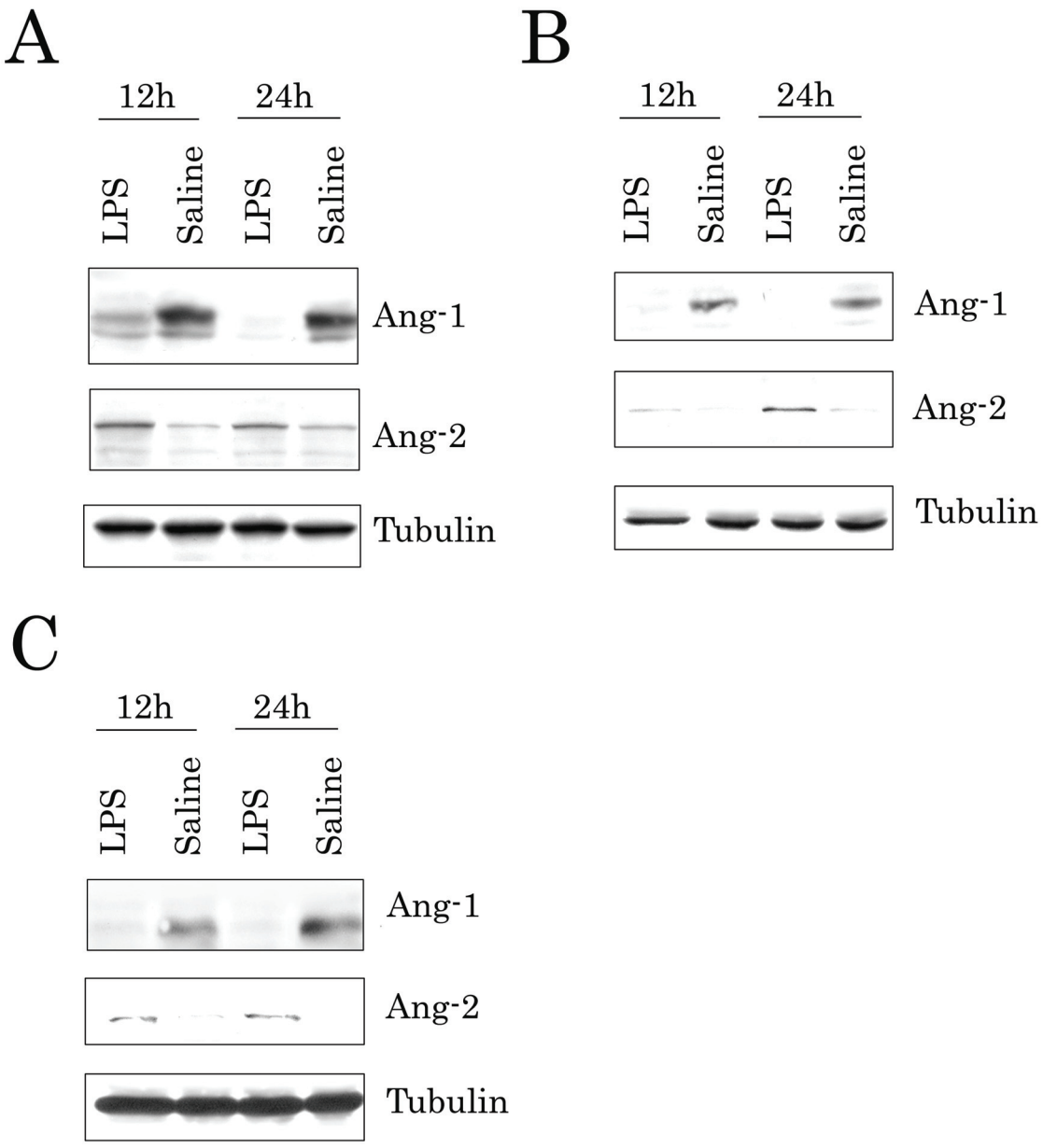

A
12h
24h
LPS
Saline
LPS
Saline
Ang-1
Ang-2
Tubulin
B
12h
24h
LPS
Saline
LPS
Saline
Ang-1
Ang-2
Tubulin
C
12h
24h
LPS
Saline
LPS
Saline
Ang-1
Ang-2
Tubulin

Figure 2

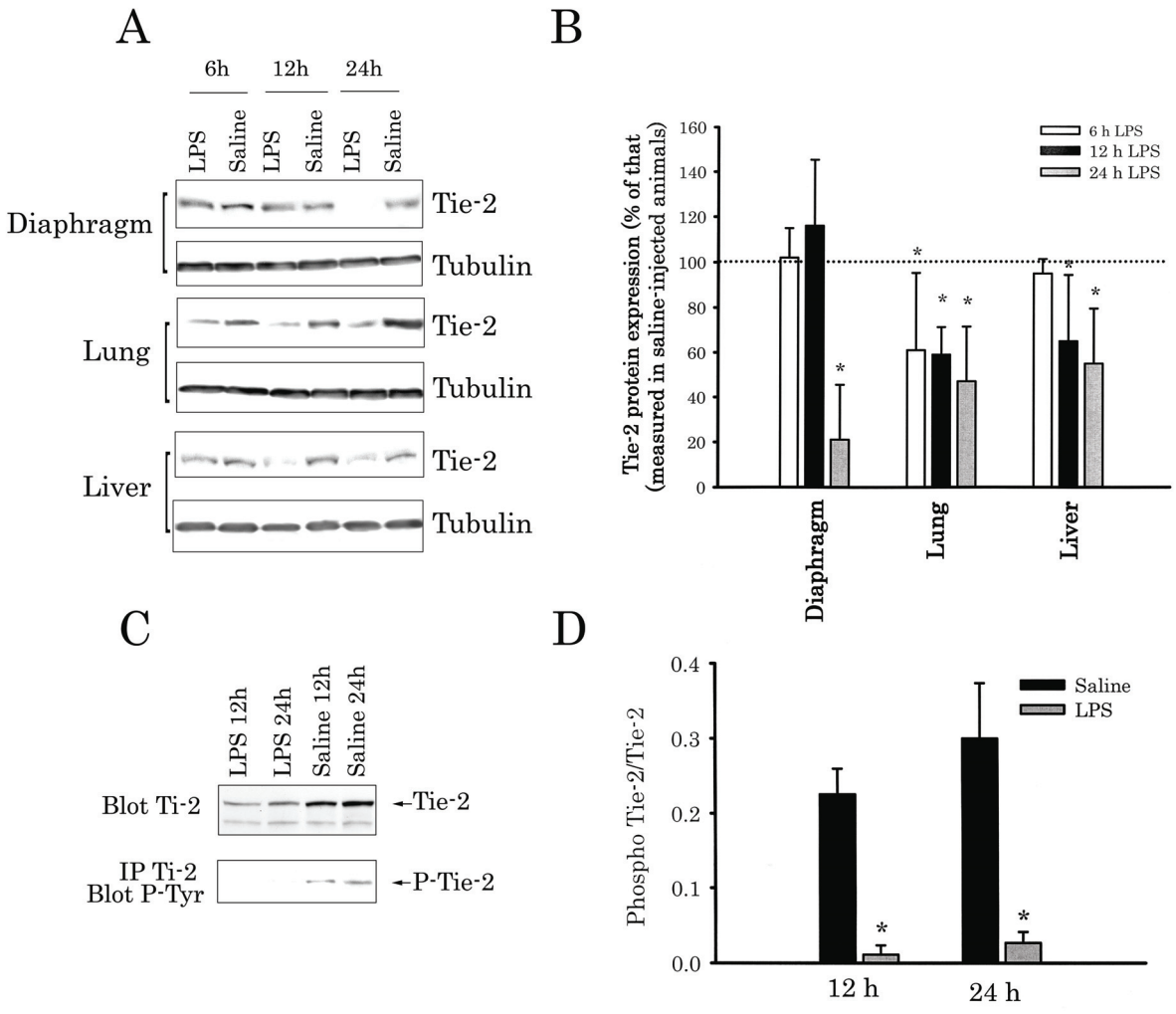

A
6h
12h
24h
LPS
Saline
LPS
Saline
LPS
Saline
Diaphragm
Tie-2
Tubulin
Lung
Tie-2
Tubulin
Liver
Tie-2
Tubulin
B
Tie-2 protein expression (% of that (measured in saline-injected animals)
160
140
120
100
80
60
40
20
0
6 h LPS
12 h LPS
24 h LPS
Diaphragm
Lung
Liver
C
LPS 12h
LPS 24h
Saline 12h
Saline 24h
Blot Ti-2
←Tie-2
IP Ti-2
Blot P-Tyr
←P-Tie-2
D
Phospho Tie-2/Tie-2
0.4
0.3
0.2
0.1
0.0
Saline
LPS
12 h
24 h

Figure 3

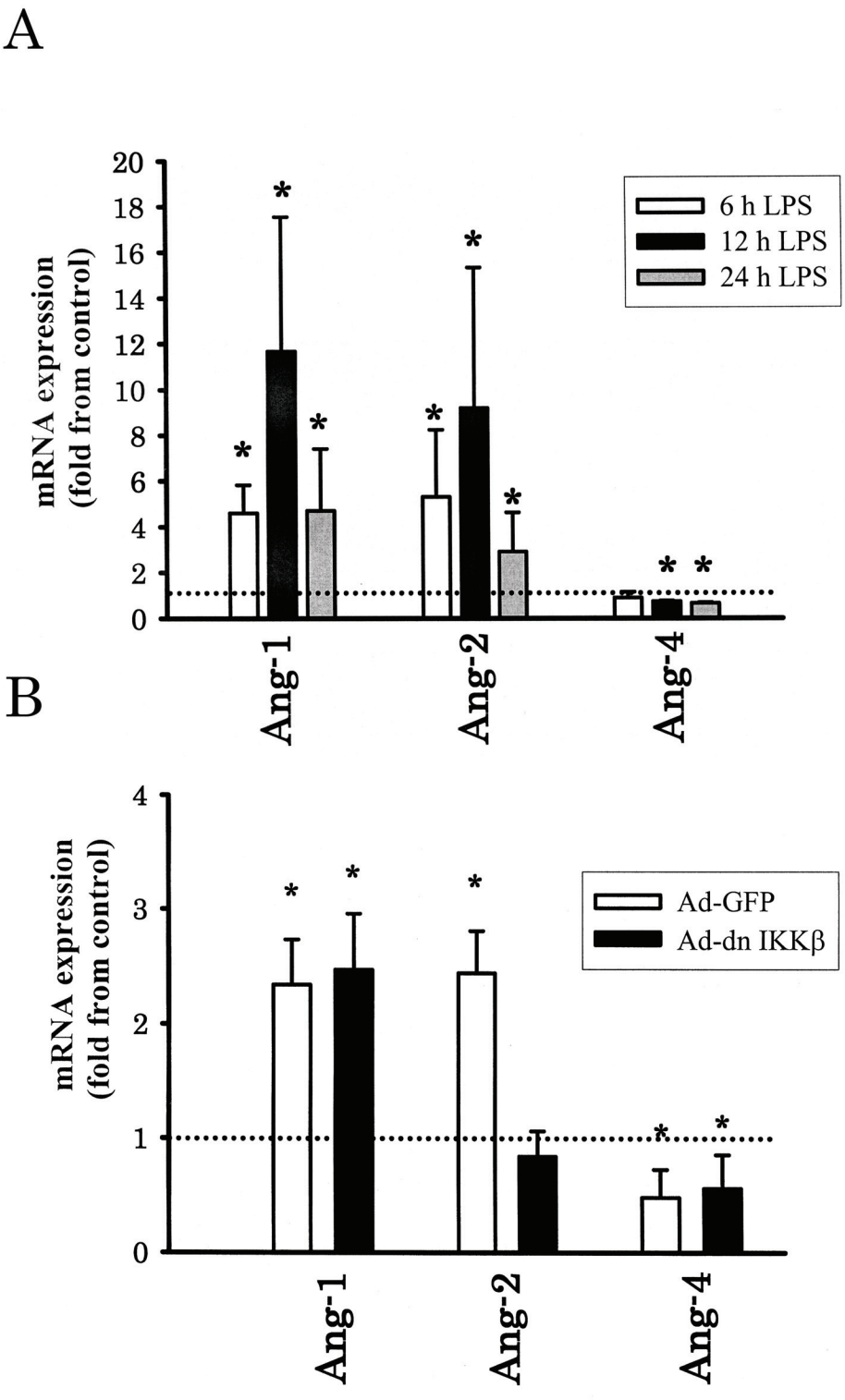
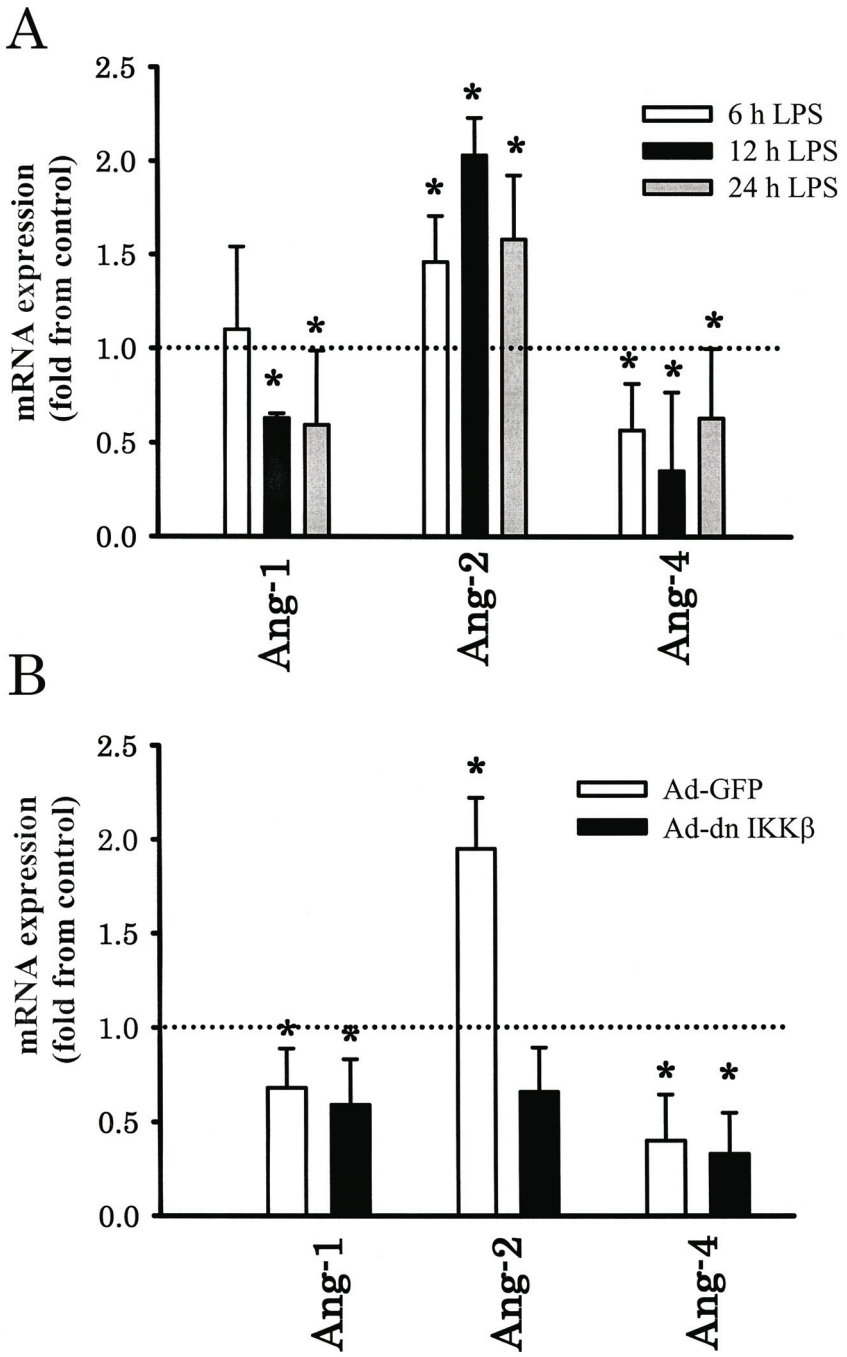

A
mRNA expression
(fold from control)
20
18
16
14
12
10
8
6
4
2
0
6 h LPS
12 h LPS
24 h LPS
Ang-1
Ang-2
Ang-4
B
mRNA expression
(fold from control)
4
3
2
1
0
Ad-GFP
Ad-dn IKKβ
Ang-1
Ang-2
Ang-4

Figure 4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mRNA expression
(fold from control)
2.5
2.0
1.5
1.0
0.5
0.0
6 h LPS
12 h LPS
24 h LPS
Ang-1
Ang-2
Ang-4
B
mRNA expression
(fold from control)
2.5
2.0
1.5
1.0
0.5
0.0
Ad-GFP
Ad-dn IKKβ
Ang-1
Ang-2
Ang-4

Figure 5

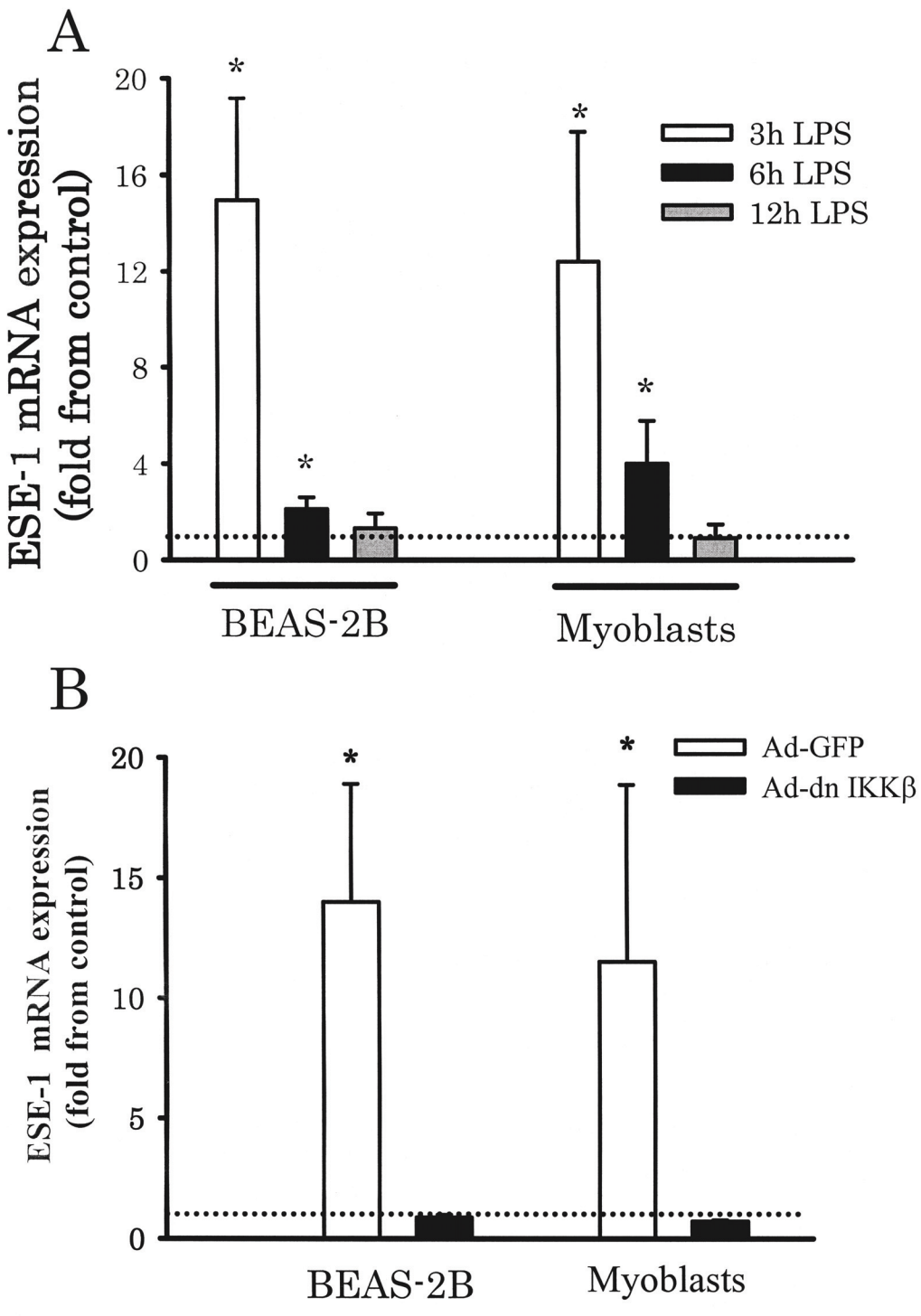
A
ESE-1 mRNA expression
(fold from control)
20
16
12
8
4
0
*
*
*
*
3h LPS
6h LPS
12h LPS
BEAS-2B
Myoblasts
B
ESE-1 mRNA expression
(fold from control)
20
15
10
5
0
*
*
Ad-GFP
Ad-dn IKKβ
BEAS-2B
Myoblasts

Figure 6

2.10 FIGURE LEGENDS

Figure 1: Changes in the expression of Ang-1, Ang-2, Ang-3 and VEGF mRNA in the lung, liver and diaphragm of mice injected with *E. coli* LPS and sacrificed 6, 12 and 24hrs later. Results are expressed as fold changes from those measured in mice injected with saline and sacrificed at the same time. *P<0.05 compared with those injected with saline. Note the substantial induction of Ang-2 mRNA while Ang-1 and Ang-3 mRNA levels declined significantly.

Figure 2: Changes in the protein levels of Ang-1 and Ang-2 in the lung (A), liver (B) and diaphragm (C) measured after 12 and 24hrs of *E. coli* LPS or saline administration in mice. Tubulin detection was used to evaluate equal protein loading. Note the decline in Ang-1 protein levels whereas Ang-2 protein expression increased significantly in response to LPS administration.

Figure 3: A & B: Representative samples and mean values of Tie-2 protein levels in the diaphragm, liver and lung measured after 6, 12, and 24 hrs of *E. coli* LPS and saline administration. *P<0.05 compared with animals injected with saline.

C: Total and tyrosine phosphorylated Tie-2 protein levels in the lungs of mice injected 12 and 24h earlier with saline or E. coli LPS.

D: Mean± SD of tyrosine phosphorylated/total Tie-2 ratios of lung samples obtained after 12 and 24 hrs of saline and *E. coli* LPS administration in mice. *P<0.05 compared with saline-injected animals.

Figure 4: A: Expression of Ang-1, Ang-2 and Ang-4 mRNAs in BEAS-2B epithelial cells measured after 6, 12 and 24 hrs of *E. coli* LPS exposure. Results are expressed as fold changes from values measured after 6, 12 and 24 hrs exposure to PBS (control). * P<0.05 compared with control.

B: BEAS-2B cells were infected with adenoviruses expressing GFP or a dominant negative form of IKKβ and were then exposed to PBS (control) or *E. coli* LPS. Expression of Ang-1, Ang-2 and Ang-4 mRNA levels in these cells were the measured with real-time PCR and expressed as fold changes from PBS-exposed (control) cells. *P<0.05 compared with control cells. Note that infection with adenoviruses expressing a dominant negative form of IKKβ had no influence on LPS-triggered changes in Ang-1 and Ang-4 levels but completely blocked LPS-induced Ang-2 expression.

Figure 5: A: Expression of Ang-1, Ang-2 and Ang-4 mRNAs in primary human skeletal myoblasts measured after 6, 12 and 24 hrs of *E. coli* LPS exposure. Results are expressed as fold changes from values measured after 6, 12 and 24 hrs exposure to PBS (control). * P<0.05 compared with control.

B: Primary human skeletal myoblast cells were infected with adenoviruses expressing GFP or a dominant negative form of IKKβ and were then exposed to PBS (control) or *E. coli* LPS. Expression of Ang-1, Ang-2 and Ang-4 mRNAs in these cells were the measured with real-time PCR and expressed as fold changes from PBS-exposed (control) cells. *P<0.05 compared with control cells. Note that

infection with adenoviruses expressing a dominant negative form of IKKβ had no influence on LPS-triggered changes in Ang-1 and Ang-4 levels but completely reversed LPS-induced Ang-2 expression.

Figure 6: A: Time course of ESE-1 transcription factor mRNA expression in BASES-2B epithelial cells and primary skeletal myoblasts after 3, 6 and 12 hrs of *E. coli* LPS exposure. Results are expressed as fold changes from values measured in cells exposed for 3, 6 and 12 hrs to PBS (control). * $P<0.05$ compared with control.

B: BEASE-2B epithelial cells and primary human skeletal myoblast cells were infected with adenoviruses expressing GFP or a dominant negative form of IKKβ and were then exposed to PBS (control) or *E. coli* LPS. Expression of ESE mRNA in these cells was then measured with real-time PCR and expressed as fold changes from PBS-exposed (control) cells. *$P<0.05$ compared with control cells. Note that infection with adenoviruses expressing a dominant negative form of IKKβ completely blocked LPS-induced ESE-1 expression.

PREFACE TO CHAPTER 3

Patients with chronic obstructive pulmonary disease (COPD) develop skeletal muscle dysfunction which is manifested as reduction in strength and endurance and poor oxygen consumption during exercise. A major determinant of skeletal muscle oxygen consumption is oxygen delivery which, in turn, is determined in part by muscle capillary density. Capillary formation in skeletal muscles is the final outcome of the balance between angiogenesis factors such as vascular endothelial growth factor (VEGF) and angiopoietins and angiostatic factors such as endostatin and angiostatin. It has been suggested that reduction of skeletal muscle capillary density and poor oxygen consumption in COPD patients are due to reduced production of angiogenesis factors such as VEGF and angiopoietins. In chapter 3 I will test this possibility by measuring the expressions of VEGF, Ang-1 and Ang-2 in limb muscles of COPD patients and control subjects. My pilot experiments suggest that Ang-2 expression is significantly elevated in limb muscles of COPD patients compared to control subjects and that this expression correlates with the severity of pulmonary disease.

Skeletal muscle regenerative capacity is largely dependent on the ability of skeletal satellite cells to initiate the myogenesis program which consists of activation, proliferation, migration and finally differentiation of satellite cells into myotubes. There is evidence that satellite cell regenerative program is depressed in limb muscles of COPD patients and that these patients have reduced capacity to recover from muscle injury. One possible cause of depressed myogenesis in satellite cells of COPD patients is elevated Ang-2 production. In chapter 3 I will evaluate this proposal by measuring the influence of Ang-2 on survival, proliferation, migration and

differentiation of human satellite cells and I will also identify the mechanisms through which Ang-2 alters the regenerative program of satellite cells.

CHAPTER 3

ANGIOGENIC FACTORS IN LIMB MUSCLES OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE PATIENTS: ROLES OF ANGIOPOIETIN 2

ANGIOGENIC FACTORS IN LIMB MUSCLES OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE PATIENTS: ROLES OF ANGIOPOIETIN 2

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3.1 ABSTRACT

Background: Skeletal muscle dysfunction contributes significantly to reduced exercise capacity in patients with chronic obstructive pulmonary disease (COPD). Reduced capillary density and poor angiogenesis have been proposed as factors causing muscle dysfunction in these patients. Angiopoietin-1 (ANGPT1) and angiopoietin-2 (ANGPT2) are angiogenesis factors that modulate endothelial cell survival but their effect on skeletal satellite cell stability and differentiation is unknown. In this study, we evaluated the expression of ANGPT1 and ANGPT2 in limb muscles of COPD patients. We also identified the factors that regulate their expression and evaluated their influence on satellite cell myogenesis.

Methodology/Principle Findings: Biopsies of *vastus lateralis* muscle were obtained from 14 control subjects and 16 patients with moderate COPD. Expression of ANGPT1 and ANGPT2 mRNA levels were quantified with real-time PCR. Satellite cell proliferation, survival, migration and differentiation were measured in primary human skeletal myoblasts. We found that mRNA levels of ANGPT2 but not those of ANGPT1 are significantly elevated in the *vastus lateralis* of COPD patients and that ANGPT2 levels correlate negatively with FEV1 and positively with muscle wasting and TNFα levels. In cultured skeletal myoblasts, ANGPT2 expression is induced by H_2O_2, but not by TNFα, IL1β and IL6. ANGPT2 significantly enhances myoblast survival and differentiation to myotubes but has no influence on proliferation and migration. Microarray analysis reveals that ANGPT2

upregulates genes involved in the regulation of cell survival, protein synthesis, glucose uptake and free fatty oxidation.

Conclusion/Significance: This first report of angiopoietin expression in COPD patients demonstrated that ANGPT2 expression is significantly upregulated in limb muscles of these patients and that this response is mediated by oxidative stress rather than pro-inflammatory cytokines. We also identified a positive role for ANGPT2 in promoting myoblast myogenesis suggesting that enhanced ANGPT2 expression may represent a positive adaptive response designed to facilitate angiogenesis and muscle fiber repair.

Key words: COPD, skeletal muscles, angiogenesis, angiopoietins, myogenesis, cytokines.

3.2 INTRODUCTION

Marked reductions in limb muscle strength and endurance are important manifestations of COPD that significantly contribute to the compromised lifestyles experienced by patients with the disease [1]. Morphologically, limb muscles exhibit wasting, shifts in fiber type distribution that result in an increase in type II fibers, altered mitochondrial enzyme activity, and reduced muscle O_2 uptake [2–5]. In addition, capillary to muscle fiber ratios in limb muscles of COPD patients have been reportedly to be significantly reduced [2]. Skeletal muscle perfusion and O_2 uptake are dependent on size of the capillary-fiber interface which is regulated by complex interactions between pro-angiogenic factors such as vascular endothelial cell growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs) and angiopoietins, which promote angiogenesis and capillary network stability, and angiostatic factors, which inhibit angiogenesis [6]. Among these, only the VEGF family of growth factors has been evaluated in limb muscles of COPD patients. Significant elevations of VEGFA and VEGFB mRNA levels have been observed in the tibialis anterior muscle of patients with moderate COPD [7]. In comparison, relatively low levels of VEGFA protein have been found in the *vastus lateralis* muscles of patients with severe COPD, as compared to control subjects [3]. No information is as yet available regarding the expression levels of other angiogenic factors in skeletal muscles of COPD patients. More recently, angiopoietins have emerged not only as angiogenesis regulators but important modulator of skeletal muscle function through their specific effects on satellite cell survival and adhesion [8].

Angiopoietins are ligands for TIE2 receptors with angiopoietin-1 (ANGPT1) being the main ligand which is produced mainly by cardiac, skeletal and smooth muscle cells and adventitial cells and to lesser extent by endothelial cells [9]. By comparison, angiopoietin-2 (ANGPT2) is expressed in the endothelium, tumor cells, macrophages and muscle cells [10–13]. In endothelial cells, ANGPT2, like ANGPT1, binds to TIE2 receptors with similar affinity to that of angiopoietin 1 (ANGPT1). However, whereas ANGPT1 is a strong agonist of TIE2 receptors, the ability of ANGPT2 to activate TIE2 receptors is highly dependent on the cell type and context [9]. Little is known about the expression of ANGPT1 and ANGPT2 in skeletal muscles of patients with COPD and whether these angiogenesis factors are produced in concert with other angiogenesis factors such as VEGF. The first objective of our study, therefore, is to evaluate the expression of ANGPT1 and ANGPT2 in limb muscles of patients with COPD and to identify the mechanisms behind the regulation of this expression. We hypothesize that these proteins are upregulated in limb muscles of COPD patients and that pro-inflammatory cytokines such as tumor necrosis factor (TNFα) and reactive oxygen species (ROS) are involved in the regulation of skeletal muscle-derived angiopoietin production in patients with COPD. This hypothesis is based on the observations that expression of angiopoietins particularly that of ANGPT2 is significantly elevated under conditions of systemic inflammation [14,15] and that serum ANGPT2 levels are induced in COPD patients during acute exacerbations of COPD [16].

Skeletal satellite cells are muscle-specific progenitors that play a critical role in skeletal muscle regeneration in response to injury. These cells proliferate, migrate, then fuse to each other and differentiate to form myofibers, leading to complete regeneration of muscle fibers. Little information is yet available regarding the regenerative capacity of skeletal muscles of COPD patients in response to injury. Martinez-Llorens *et al*. [17] have described the presence of microstructural damage in the intercostal muscles of COPD patients and that this muscle injury was associated with increased satellite cell activation suggesting the presence of active muscle regeneration program (myogenesis) designed to repair damaged muscle fibers in COPD patients. Many angiogenic growth factors such as VEGF, FGFs, and insulin-like growth factor-1 (IGF1) act in an autocrine fashion to promote satellite cell myogenesis by enhancing proliferation, migration, differentiation and survival of these cells [18–20]. Although the vascular effects and the signaling of angiopoietins in endothelial cells are well characterized, there is no information yet regarding the influence of angiopoietins on the myogenesis program of skeletal muscle satellite cells. The second objective of this study, therefore, is to evaluate the functional effects of angiopoietins on skeletal satellite cell myogenesis including proliferation, migration, differentiation and survival and to investigate the mechanisms through which angiopoietins influence these processes. On the basis of preliminary experiments, we hypothesize that angiopoietins play positive roles in promoting both differentiation and survival of muscle cells and that these effects are mediated through selective activation of specific cellular signaling pathways that include protein kinase B (AKT) and the ERK1/2 members of the mitogen-activated protein kinases (MAPKs). Our results indicate that ANGPT2 and not

ANGPT1 is expressed to significantly higher levels in limb muscles of COPD patients and that this expression is related to oxidative stress. Our results also indicate that ANGPT2 exerts positive effects on satellite cell myogenesis program and that this effect is mediated through activation of multiple signaling pathways.

3.3 MATERIALS AND METHODS

3.3.1 EXPRESSION OF ANGIOPOIETINS IN HUMAN VASTUS LATERALIS MUSCLE

Patient selection: Written informed consent was obtained from all subjects. All procedures have been approved by the Ethics Committees of Laval University and have been conducted according to the principles expressed in the Declaration of Helsinki. Sixteen patients with COPD and 14 control subjects were recruited for this study. All subjects were classified as sedentary according to a physical activity questionnaire adapted for older and retired subjects [21]. COPD diagnosis was established using Global Initiative for Chronic Obstructive Lung Disease guidelines [22]. Exclusion criteria included chronic respiratory failure, bronchial asthma, coronary artery disease, neuromuscular disease, chronic metabolic disease and/or treatment with drugs known to alter muscle structure and function. No patient was receiving long-term O_2 therapy. Height and weight were measured according to standardized methods. Arterial blood was drawn from a radial artery and blood gases were analyzed with a blood gas analyzer (AVL 995, AVL Scientific, Roswell, GA). Forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were measured using a pneumotachograph and standard methods. The measured values were expressed as a % of predicted value, according to age, height, and weight. Mid-thigh muscle cross-sectional area (CSA) was determined using computed tomography of the right and left thighs, halfway between the pubic symphysis and the inferior condyle of the femur, using a fourth-generation Toshiba scanner (900S, Toshiba Inc., Tokyo, Japan). In all control subjects and COPD patients needle biopsies of the *vastus lateralis* muscle were performed at mid-thigh,

as described by Bergström [23] and tissues were immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from quadriceps muscle biopsies using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Oakville, ON). Quantification and purity of total RNA was assessed by A260/A280 absorption.

Real-time PCR detection of gene expression in *vastus lateralis* muscle: We first evaluated whether ANGPT1, ANGPT2 and VEGF mRNA levels in the *vastus lateralis* muscle biopsies were different among control subjects and COPD patients. To investigate whether pro-inflammatory cytokine levels correlate with the expression of these angiogenesis factors, we also measured mRNA levels of tumor necrosis factor (TNFα), interleukin-1 (IL1β) and interleukin-6 (IL6) using real-time PCR. RNA (2μg) was reverse- transcribed using Superscript II® Reverse Transcriptase Kits and random primers (Invitrogen Inc.). Reactions were incubated at 42°C for 50min and at 90°C for 5min. Real-time PCR was performed using a Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). To quantify expressions of human ANGPT1, ANGPT2, VEGF, TNFα, IL1β and IL6 mRNA transcripts and 18S (endogenous control), TaqMan® Gene Expression Assays (Applied Biosystem Inc.) specific to these genes were used. The thermal profile was as follows: 95°C for 10min and 40 cycles of 95°C for 15sec, 57°C for 30sec, and 72°C for 33sec. All real-time PCR experiments were performed in triplicate. A melt analysis for each PCR experiment was performed to assess primer-dimer formation or contamination. To determine the absolute copy numbers of ANGPT1, ANGPT2, VEGF, TNFα, IL1β, IL6 and 18S mRNA transcripts in

human muscle samples, standard curves that related the cycle threshold (C_T) values of these genes to the copy numbers were established, as described in our recent study [10]. Copy numbers of these genes were then normalized per 10^3 copies of 18S. In addition, relative quantification of human mRNA levels of target genes in quadriceps of COPD patients compared to those of control subjects was determined using the threshold cycle (ΔΔCT) method.

3.3.2 ANGPT2 EXPRESSION IN SKELETAL AND ENDOTHELIAL CELLS

We focused in subsequent sections of this study on evaluating the expression and the biological effects of ANGPT2 on skeletal precursor cells because our results revealed that ANGPT2 and not ANGPT1 is expressed in significantly higher levels in *vastus lateralis* muscle biopsies in COPD patients compared with control subjects (see below). While ANGPT2 is produced mainly by endothelial cells, production of this factor by primary skeletal precursor cells is less clear. Hence, we measured in this section the levels of ANGPT2 production in both, human and murine primary skeletal precursor cells. Primary human muscle precursor cells (human myoblasts) immortalized by expression of the E6E7 early region from human papillomavirus type 16, were generously provided by Dr. E. Shoubridge (McGill University, Montréal, QC), and cultured in SkBM culture medium (SkBM® Bullet Kit, Cambrex, East Rutherford, NJ) supplemented with 15% inactivated fetal bovine serum (FBS)[24]. Cells were collected and total RNA was extracted as described above. The levels of ANGPT2 mRNA and 18S (control gene) were detected using TaqMan real-time PCR assays (Applied Biosystems Inc.) as described above. Results were analyzed by using the comparative threshold cycle (C_T) and are also expressed in copy number normalized per copies of 18S as

described above. To evaluate the changes in ANGPT2 mRNA in human myoblasts during differentiation to myotubes, human skeletal myoblasts were induced to differentiate into myotubes by growing confluent myoblasts (90% confluent) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Inc., Carlsbad, CA) supplemented with 2% inactivated horse serum (HS) for 7 days. Cells were collected at days 0 (myoblasts), 1, 3, 5 and 7 and total RNA was extracted as described above. ANGPT2 mRNA and 18S levels during differentiation were detected using real-time PCR as described above.

All animal experiments were approved by McGill University Animal Care Committee (protocol# 3870). To isolate primary murine skeletal precursor cells, tibialis anterior muscle strips were extracted from 6-week-old C57/BL6 mice, digested with collagenase (0.2% at 37°C for 60 min) and then triturated to break muscle tissues into single fibers. Individual fibers were washed in (DMEM) and phosphate-buffered saline, transferred into Matrigel®-coated (1mg/ml in DMEM) 6-well plates and maintained in DMEM supplemented with 1% penicillin/streptomycin and 0.2% amphotericin B, 10% HS and 0.5% chick embryo extract (MP Biomedicals, Aurora, OH) for 4 days, which allowed myoblasts to attach to the substratum. Cells were then collected and total RNA was extracted as described. ANGPT2 mRNA levels were detected using real-time PCR and murine Taqman® ANGPT2 assays (Applied Biosystems). ANGPT2 mRNA expression in these cells was expressed as copies normalized for 18S copies.

Regulation of ANGPT2 expression in human myoblasts by cytokines and H_2O_2: To assess whether muscle-derived ANGPT2 production is under the control

of inflammatory cytokines and oxidative stress, we measured the effects of TNFα, IL1β, IL6 and hydrogen peroxide (H_2O_2, mediator of oxidative stress) on ANGPT-2 expression in muscle cells. Human skeletal myoblasts were plated into 12-well plates (10^5 cells per well) and maintained for 24h in SkBM culture medium containing 5% FBS. Cells were then stimulated with recombinant human IL-1β (20 ng/ml), TNFα (40 ng/ml), IL6 (50 ng/ml), mixture of the three cytokines, H_2O_2 (0.5 mM), or a control solution of phosphate-buffered saline (PBS). Cells were collected 24h later and prepared for total RNA extraction and ANGPT2 mRNA expression using real-time PCR (see above). Relative quantification of ANGPT2 mRNA levels in cells treated with cytokines or H_2O_2 compared to control cells was determined using the threshold cycle method. In additional experiments, human myoblasts were stimulated for 24h with TNFα (40 ng/ml) or H_2O_2 (0.5 mM) or PBS. Media and cell lysates were collected ANGPT2 protein levels in the media were measured with ELISA (Angiopoietin-2 Quantikine ELISA, R& D Systems Inc.) and normalized for cell lysate protein levels.

Regulation of ANGPT2 expression in human umbilical vein endothelial cells (HUVECs): To compare the influence of ANGPT2 on survival of skeletal precursor cells to that elicited in endothelial cells, we studied HUVECs which were cultured in endothelial basal medium (MCDB131) supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplement, 2mM glutamine, heparin, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). HUVECs were plated into 12-well plates (10^5 cells per well) and maintained for 24h in endothelial cell basal medium supplemented with 2% FBS

containing PBS (control), TNFα (40 ng/ml) or H_2O_2 (0.3 mM). Cells and media were then collected and ANGPT2 protein levels in the media were measured with ELISA as described above.

3.3.3 REGULATION OF MYOBLAST MYOGENESIS BY ANGPT2

Myoblast proliferation: The effects of ANGPT2 on myoblast cell proliferation were measured by seeding human myoblasts (1 x 10^5 cells) into 6-well culture plates for 24h in SkBM culture medium containing 15% FBS. Culture medium was then replaced with SkBM culture medium containing 5% FBS and either PBS or 150, 300 and 600 ng/ml of recombinant human ANGPT2. These concentrations have been extensively employed to evaluate the biological responses to ANGPT2 in cultured cells. Culture medium containing PBS or ANGPT2 protein was replenished every 24h. After 72h of exposure, cells were then exposed to 0.5% trypsin-EDTA, stained with trypan blue and viable cells were counted by hematocytometer.

Myoblast migration: Regulation of skeletal myoblast migration by ANGPT2 was assessed using wound healing assay. Human myoblasts (1 x 10^5 cells) were seeded into 6-well culture plates and maintained for 24h in SkBM culture medium containing 15% FBS. Cells were then carefully wounded using a 200-µl pipette tip, as previously described [25]. Cellular debris was removed by washing with phosphate-buffered saline (PBS). After wounding, culture medium was replaced with SkBM containing 5% FBS and either PBS (control) or 600 ng/ml recombinant human ANGPT2. Wounds were photographed immediately after wounding (time = 0) and 12 and 18h later using an Olympus inverted microscope (Model X70) equipped with phase-contact lenses. Migration was evaluated by measuring the

reduction in the diameter of the wound after migration of the cells into the cell-free zone [3726}. For each condition, three wells of a given 6-well plate were used, and the procedure was performed in triplicate.

Intracellular signaling by ANGPT2 in satellite cells: Several reports have confirmed that the PI-3 kinase/AKT and the Erk1/2 pathways are important in promoting many of the biological effects of angiopoietins in endothelial cells [26,27]. To evaluate whether these two pathways are activated by ANGPT2 in skeletal muscle precursors, human skeletal myoblasts were maintained in SkBM culture medium containing 5%FBS for 12h. Cells were then stimulated with PBS (control) or recombinant human ANGPT2 (300 ng/ml) for 5, 15, 30 and 60 min. The medium was then removed and adherent cells were washed twice with PBS and lysed in basic lysis buffer (see below). In addition, human myoblasts were stimulated for 15 min with PBS or 50, 150, 300 or 600 ng/ml of ANGPT2. Cells were lysed in basic lysis buffer containing HEPES (50mM), NaCl (150mM), NaF (100mM), EDTA (5mM) and protease inhibitors (aprotinin 5mg/ml, leupeptin 2mg/ml and PMSF 100mM). Cell debris and nuclei were separed by centrifugation at 14,000g for 5min at 4°C. Supernatants were then boiled for 5min then loaded onto tris-glycine SDS-PAGE. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked for 1h with 5% nonfat dry milk, and incubated overnight at 4°C with primary antibodies to phospho-AKT (Thr^{308}), total AKT, phospho-Erk1/2 (Thr^{202}/Tyr^{204}) and total Erk1/2 (Cell Signaling Inc.). Proteins were detected with horseradish peroxidase–conjugated secondary antibodies and ECL reagents (Chemicon). Loading of equal amounts of protein was

confirmed by stripping the membranes and re-probing with anti-tubulin antibody (Sigma-Aldrich). Blots were scanned with an imaging densitometer, and optical densities of protein bands were quantified with ImagePro software (Media Cybernetics, Carlsbad, CA). Predetermined molecular weight standards were used as markers. Protein concentrations were measured by the Bradford method, with BSA as a standard.

Myoblast survival and apoptosis: To evaluate the influence of ANGPT2 on skeletal muscle precursor cell survival, human skeletal myoblasts ($25x10^3$ cells) were seeded into 96-well plates and maintained for 12h in SkBM culture medium containing 15%FBS. Culture medium was then replaced with SkBM culture medium (0%FBS) containing either PBS or 300 ng/ml recombinant human ANGPT2. Cell cytotoxicity and caspase3/7 activity were measured 36h later using CytoTox-Fluor™ Cytotoxicity Assay and Caspase-Glo™ 3/7 Assay, respectively, according to the manfacturer's instructions (Promega Inc. Madison, WI). The involvement of the PI-3 kinase/AKT and Erk1/2 pathways in the regulation of muscle cell survival and caspase 3/7 activity, we incubated human skeletal myoblasts in SkBM culture medium (0%FBS) containing either PBS (control condition) or 300 ng/ml of recombinant human ANGPT2 in the presence and absence of 2 μM of PD184352 (Erk1/2 inhibitor), 50 nM of wortmannin (PI-3 kinase inhibitor) and 1 μM of API-2 (Tricirbine, AKT inhibitor). Cell death (cytotoxicity) and caspase3/7 activity were measured 36h later using CytoTox-Fluor™ Cytotoxicity Assay and Caspase-Glo™ 3/7 Assay, respectively.

Myoblast differentiation: We evaluated the influence of ANGPT2 on human myoblast differentiation by over-expressing ANGPT2 in human skeletal myoblasts using adenoviruses. The choice of using adenoviruses to deliver ANGPT2 was aimed at achieving sustained endogenous elevation of ANGPT2 production over several days required for full differentiation of skeletal myoblasts into myotubes. Recombinant adenoviruses expressing ANGPT2 (Ad-ANGPT2) were constructed using the Adeno-Quest™ system (Quantum Biotechnology Inc., Montreal, Canada). Full-length cDNA encoding human ANGPT2 was cloned into shuttle vector pQBI-AdCMV5GFP [28]. Sub-confluent human myoblasts were maintained for 6h in SkBM culture medium containing 250 MOI (multiplicity of infection) of Ad-ANGPT2 or Ad-GFP (control cells). Cells were then washed and maintained for 48h in SkBM culture medium containing 15% FBS to reach full confluence. After this recovery period, the cells were collected (day 0, myoblast phase) for RNA extraction and also differentiated into myotubes by replacing the medium with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Inc., Carlsbad, CA) supplemented with 2% inactivated horse serum (HS). Differentiating cells were collected at 1, 3, 5 and 7 days of incubation with the differentiation medium. Total RNA was extracted as described above. Total RNA (2μg) was then reverse transcribed using Superscript II® Reverse Transcriptase Kits and random primers (Invitrogen). Reactions were incubated at 42°C for 50min and at 90°C for 5min. Expression of myogenic transcription factors MyoD and myogenin as well as muscle-specific myosin heavy chain and creatine kinase mRNA levels as well as that of 18S levels during differentiation were detected using real-time PCR as

described above. Primers designed to amplify these transcripts are listed in Table S1. Real-time PCR was performed using a Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). To determine the absolute copy numbers of mRNA transcripts of a specific gene and 18S mRNA transcripts, standard curves that related the cycle threshold (C_T) values of these genes to the copy numbers were established, as described in our recent study [10]. Copy numbers of these genes were then calculated as described above and normalized per copies of 18S.

3.3.4 MECHANISMS OF ANGPT2 ACTION IN SKELETAL MYOBLASTS

To investigate the mechanisms through which ANGPT2 regulates skeletal myoblast survival and differentiation, we over-expressed human ANGPT2 in human skeletal myoblasts using adenoviruses (Ad-ANGPT2) and compared mRNA expression profiles of these cells to those infected with control adenoviruses (Ad-GFP). Sub-confluent human myoblasts were maintained for 6h in SkBM culture medium containing 250 MOI (multiplicity of infection) of Ad-ANGPT2 (n=6) or Ad-GFP (n=6). Cells were then washed and maintained for 48h in SkBM culture medium containing 15% FBS. After this recovery period, the culture medium was changed to SkBM culture medium containing 5% FBS. Cells were collected 12h later and total RNA was extracted as described above.

Illumina microarrays: Total RNA (100 ng) was amplified using the Illumina RNA Amplification kit (Illumina Inc., San Diego CA) and labelled by incorporation of biotin-16-UTP. Samples were hybridized to Sentrix Genome-Wide Expression BeadArray™ (Illumina Inc.). These arrays use beads containing 50-mer gene-specific probes (a total of 46,000 probes per array). Arrays were scanned with an Illumina BeadArray Reader (Montreal Genome Centre, Montreal, Canada)

and data processing and normalization were performed using Illumina Bead-Studio software. Signal values were normalized by global mean and log transformed using GeneSifter software (VizX Labs, Seattle, WA, USA). Pairwise comparisons and ANOVA were subsequently performed using FlexArray 1.1.3 sofware package (Genome Quebec, Montreal, Quebec, Canada) and a difference of at least two-fold with a P-value of less than 0.05 was considered as a statistically significant change in gene expression. All data is MIAME compliant and the raw data has been deposited in a MIAME compliant database (GEO). To investigate gene networks regulated by ANGPT2 in cultured human skeletal myoblasts, we analyzed the microarray results using Ingenuity Pathway Analysis which is a web-based bioinformatics tool (IPA, http://www.ingenuity.com) based on >1.7 Million published articles. IPA is a knowledge database generated from the peer-reviewed scientific publications that enables discovery of biological networks in gene expression data, determining the functions most significant to those networks. Gene name identifiers or Ingenuity probe set ID's were uploaded into IPA and queried against all other genes stored in the IPA knowledge database. Each Ingenuity probe set ID was mapped to its corresponding gene identifier in the IPA knowledge database. Probe sets representing genes having direct interactions with genes in the IPA knowledge database are called ‘‘focus’’ genes, which were then used as a starting point for generating functional networks. Each generated network is assigned a score according to the number of differentially regulated focus genes in our array dataset. These scores are derived from negative logarithm of the P indicative of the likelihood that focus genes found together in a network due to

random chance. Scores of 14 or higher have 99.9% confidence level of significance. In reporting our findings we list networks with a substantially higher confidence limit and thus represent strong evidence for a given biological pathway being regulated by ANGPT2 in human skeletal myoblasts. It should be noted however that while the database extends the interpretation beyond mRNA transcript levels (as network genes don't have to be differentially expressed at the mRNA level) the database is finite and reflects current knowledge.

To confirm the results obtained with the microarrays, we performed real-time PCR to evaluate the expression of four genes which were found to be upregulated (TEL2 and LEP) and downregulated (CSF3 and ANGPTL4) in cells infected with Ad-ANGPT2 compared to cells infected with Ad-GFP. Real-time PCR was performed as described above using primers listed in Table S4. Relative quantification of TEL2, LEP, CSF3 and ANGPTL4 mRNA levels in cells infected with Ad-ANGPT2 compared to cells infected with Ad-GFP was determined using the threshold cycle (ΔΔCT) method. In addition, we verified enhanced leptin production in muscle cells by directly measuring leptin levels in the culture medium. Sub-confluent human myoblasts were maintained for 6h in SkBM culture medium containing 250 MOI (multiplicity of infection) of Ad-ANGPT2 (n=6) or Ad-GFP (n=6). Cells were then washed and maintained for 72h in SkBM culture medium containing 5% FBS. The levels of secreted leptin in the culture medium were measured with a commercial leptin ELISA kit from R&D Systems.

Pathways regulated by ANGPT2: Data were analyzed using the IPA (Ingenuity® Systems, www.ingenuity.com). A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each gene

identifier was mapped to its corresponding gene object. A fold-change cutoff of 2 for both up- and down-regulation and a p-value cutoff of 0.05 were set to identify the genes to be analysed. These genes, called focus molecules, were overlaid onto a global molecular network developed from information in the IPA. Networks of these focus molecules were then algorithmically generated based on their connectivity. The functional analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network. Genes and gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, textbook or canonical information stored in the IPA. Human, mouse and rat orthologs of a gene are stored as separate objects in the IPKB, but are represented as a single node in the network. The node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. It should be emphasized that one round of analyses were performed with the Ingenuity Pathway Analysis system considering both up- and downregulated genes in cells infected with Ad-ANGPT2 compared with cells infected with Ad-GFP.

3.3.5 STATISTICAL ANALYSIS

Results are expressed as means ±SE in all figures. Differences in the expression of angiogenic factors in the large group experiments were detected with two-way ANOVA followed by a Tukey test. Pearson's correlation coefficient was used to assess relationships between different variables between control subjects and patients with COPD in the large group. Differences in terms of cell proliferation, migration, cell death, caspase 3/7 activity, and muscle-specific gene mRNA levels

during the differentiation protocols were detected with unpaired t-tests with a Bonferroni-type adjustment. Values of $P<5\%$ were considered significant.

3.4 RESULTS

3.4.1 EXPRESSION OF ANGIOGENESIS FACTORS IN HUMAN *VASTUS LATERALIS* MUSCLE

Characteristics of control subjects and COPD patients who underwent *vastus lateralis* muscle biopsies are listed in Table 1. While age, weight and BMI were similar in the two groups, thigh cross-sectional areas, FEV1, FVC and FEV1/FVC ratios were significantly lower in COPD patients (Table 1). *Vastus lateralis* TNFα mRNA levels were significantly higher (0.413±0.021 vs. 0.105±0.011 copies/10^3 copies of 18S, $p<0.05$) in COPD patients. In comparison, muscle IL1β mRNA levels (3.1±1.0 vs. 3.93±1.4 copies/10^3 copies of 18S) and IL6 mRNA levels (0.046±0.01 vs. 0.038±0.01 copies/10^3 copies of 18S) were similar between control subjects and COPD patients. The expression of three angiogenesis genes was measured in the *vastus lateralis* muscle samples of this study. ANGPT2 mRNA levels averaged about 3-fold higher in COPD patients ($P<0.05$), whereas VEGFA and ANGPT1 mRNA levels were not significantly different between the two groups (Figure 1A). In this group of subjects and patients, muscle ANGPT2 mRNA levels correlated negatively with predicted FEV1% ($r=0.72$, $P<0.05$) and right thigh cross-sectional areas ($r=0.46$, $P<0.05$) (Figure 1B) but positively with muscle TNFα expression (Figure 1C).

3.4.2 ANGPT2 EXPRESSION IN SKELETAL AND ENDOTHELIAL CELLS

In primary human myoblasts ANGPT2 mRNA levels (0.24±0.04 copies/10^3 copies of 18S) are similar to those detected in murine skeletal myoblasts (0.25±0.03 copies/10^3 copies of 18S) suggesting that ANGPT2 in muscle tissues can be

derived from endothelial and muscle cells. Expression of ANGPT2 mRNA in human myoblasts is significantly induced when they differentiate into myotubes, indicating that mature muscle fibers produce higher levels of ANGPT2 than their precursors (Figure 2A). Figure 2B indicates that ANGPT2 mRNA levels in human myoblasts were significantly reduced by IL1β, significantly augmented by H_2O_2 whereas TNFα and IL6 had no effects. Lack of TNFα effect on ANGPT2 production and upregulation of this production by H_2O_2 were verified by measuring ANGPT2 protein levels in the media of human myoblasts and human umbilical vein endothelial cells (Figure 2C and D).

3.4.3 REGULATION OF MYOBLAST MYOGENESIS BY ANGPT2

To evaluate the functional significance of increased ANGPT2 expression on muscle fiber regeneration, we measured the effects of exogenous ANGPT2 on proliferation, migration, differentiation and survival of human skeletal myoblasts. ANGPT2 has no effect on myoblast proliferation and migration (Figure 3A-C). However, ANGPT2 significantly reduces cytotoxicity and capase-3/7 activity in skeletal myoblasts exposed to complete serum withdrawal, indicating that ANGPT2 promotes myoblast survival (Figure 3D). To investigate the signaling pathways through which ANGPT2 promotes myoblast survival, we evaluated the effects of ANGPT2 on activation of two important pro-survival pathways (PI3 kinase/AKT and ERK1/2). ANGPT2 elicited transient and dose-dependent increases in AKT phosphorylation on $Thre^{308}$ (site of PI3 kinase mediated phosphorylation) and ERK1/2 phosphorylation on $Thre^{202}/Tyr^{204}$ (site of ERK1/2 mediated phosphorylation (Figure 4A and B). The importance of the PI3 kinase/AKT and

ERK1/2 pathways in the pro-survival effects of ANGPT2 was assessed using selective pharmacological inhibitors of these pathways. The inhibitory effects of ANGPT2 on serum deprivation-induced cytotoxicity and caspase 3/7 activity are completely eliminated in the presence of selective inhibitors of PI3 kinase, AKT and Erk1/2 (Figure 4C and D) These results suggest that ANGPT2 promotes myoblast survival and inhibits apoptosis in these cells through the PI3 kinase/AKT and Erk1/2 pathways.

Figure 5 shows the influence of ANGPT2 on differentiation of human skeletal myoblasts. Significant inductions of MyoD and myogenin occur in control adenovirus (Ad-GFP) transfected cells incubated for 1 day in differentiation medium; creatine kinase and myosin heavy chain expressions are significantly induced after 3 and 5 days of incubation, respectively (Figure 5). In myoblasts transfected with Ad-ANGPT2, even greater levels of differentiation take place, as indicated by the earlier and significantly higher expression levels of MyoD, myogenin, creatine kinase and myosin heavy chain genes, as compared to cells infected with Ad-GFP (Figure 5).

3.4.4 MECHANISMS OF ANGPT2 ACTION IN SKELETAL MYOBLASTS

The transcriptomes of human skeletal myoblasts infected with adenoviruses expressing GFP (control) and ANGPT2 were compared using Illumina microarrays which revealed that expressions of 45 genes were significantly upregulated by ANGPT2 in skeletal myoblasts (Tables 2 and S2). The largest relative increase was detected in NRP1 (receptor for VEGF family of proteins) and TELO2 (regulator of cell cycle). ANGPT2 also induced the expression of several other genes involved in cell signaling, including LEP (leptin) (Tables 2 and S2).

Tables 2 and S3 list 95 genes whose expression is significantly downregulated by ANGPT2. Heat shock 70kDa proteins, SPANX transcription factors, inducible nitric oxide synthase (iNOS), FOS and FOBSB subunits of activating protein 1 (AP1) transcription factor and colony stimulating factor 3 are the most strongly inhibited genes. ANGPT2 also inhibited the expression of IL6, the chemokine CLL26, angiopoietin-like 4, forkhead transcription factor FOXF2 and insulin receptor substrate 4 (Tables 2 and S3).

Microarray results were verified by detecting gene expressions of two upregulated genes (TELO2 and LEP) and two downregulated genes (CSF3 and ANGPTL4) (Figure 6A). Upregulation of LEP (leptin) mRNA expression in cells infected with Ad-ANGPT2 is associated with a significant increase in secreted LEP protein (Figure 6B). Ingenuity Pathway Analysis software was used to analyze the microarray dataset in the context of biological pathways. Table S3 lists the top networks and top cellular functions that were generated (data supplement). Seven major networks of ANGPT2 regulated genes were detected which contained 24, 18, 14, 11, 10, 10, and 9 focus genes, respectively (Table 3). The top network contains several highly connected nodes, with IL6 being the most highly connected (Figure 7). The second top network contains several highly connected nodes, including two mitogen activated protein kinase pathways (p38 and SAPK/JNK), inducible nitric oxide synthase (NOS2), three immune modulators (IL1, IL12 and IFNα) and NFκB transcription factor (supplementary Figure 1). It should be noted that the majority of the genes identified in the first and second top networks were downregulated by ANGPT2.

3.5 DISCUSSION

The main findings of our study are: 1) In the *vastus lateralis* of patients with moderate COPD, ANGPT2 mRNA levels but not those of VEGF and ANGPT1 are significantly higher than control subjects; 2) Elevated ANGPT2 expression in the *vastus lateralis* of COPD patients negatively correlates with the severity of COPD but positively correlates with the degree of muscle wasting and *vastus lateralis* TNFα expression; 3) ANGPT2 expression in both, skeletal myoblasts and endothelial cells is induced by H_2O_2; 4) Upregulation of ANGPT2 expression in skeletal myoblasts is associated with increased myoblast survival and differentiation. These effects are mediated through activation of the PI3-kinase/AKT and Erk1/2 pathways and through selective and distinct changes in overall cellular gene expression profiles.

3.5.1 STUDY LIMITATIONS

Two points deserve attention regarding the experimental approach used in this study. First, we studied 16 COPD patients and 14 control subjects in terms of angiopoietin expression in *vastus lateralis* muscle biopsies. One reason for not studying even larger number of subjects is because that the *vastus lateralis* muscle biopsy is an invasive procedure, thereby restricting the number of available volunteers. Moreover, very restrictive inclusion and exclusion criteria were applied in selecting equal numbers of control subjects and COPD patients so that they would have very similar characteristics, including physical activity and nutritional statuses. Second, regulation of ANGPT2 expression in skeletal muscle cells and the functional importance of ANGPT2 in regulating myogenesis were evaluated without addressing the situation in vascular cells. This choice was made because vascular roles of ANGPT2 have been

extensively studied by many investigators but no information is as yet available regarding either ANGPT2 expression or function in skeletal muscle cells. Also, in this study, exogenous recombinant human ANGPT2 proteins were used at physiological concentrations. In the past, this has been shown to be an effective method of promoting endothelial cell survival and inhibiting apoptotic processes [29]. Another proven approach has been to expose muscle cells to ANGPT2 over prolonged periods, as during muscle differentiation, through transfection with adenoviruses expressing ANGPT2. This method has successfully been used by numerous investigators to produce sustained ANGPT2 levels in *in-vivo* and *in-vitro* settings [30,31].

3.5.2 EXPRESSION OF ANGIOGENESIS-RELATED GENES IN HUMAN VASTUS LATERLAIS

The production of angiogenic factors by skeletal muscle fibers plays very important roles in stabilizing capillary networks and in maintaining normal O_2 supply to active muscle fibers. The importance of normal capillarity to muscle performance is delineated by the strong positive relationship between peak exercise O_2 consumption and muscle capillary density [32]. In patients with moderate and severe COPD, there is no information as to whether reduced limb muscle contractile function (strength and endurance) is mediated in part through abnormalities in angiogenesis factor production. To the best of our knowledge, there have only been two studies published that evaluate the expression of VEGF family members in limb muscles of COPD patients. Barreiro *et al*. [3] reported that VEGFA protein levels are significantly lower in the *vastus lateralis* muscles of patients with severe COPD, as compared to control subjects, while Jatta *et al.* [7] found no differences in mRNA expressions of VEGFA,

VEGFB and VEGFC genes in the tibialis anterior muscles of patients with severe COPD, as compared to control subjects. The present results, which include the fact that VEGF is expressed at similar levels in the *vastus lateralis* muscles of control subjects and patients with moderate COPD, are in accordance with those of Jatta *et al*., but differ from those described by Barreiro *et al*. There is no clear explanation for the differences between our findings and those of Barreiro *et al*., however, we speculate that VEGF expression in the muscles of COPD patients may be dependent on the severity of COPD, such that patients with severe COPD included in the study of Barreiro *et al.* (average FEV1 of 33% predicted) show significant declines in VEGF expression while patients with moderate COPD, as were included in our study (average FEV1 of 42% predicted), do not.

One of the major observations of our study is that ANGPT2 expression is significantly higher in the patient group compared with control subjects. Relatively higher ANGPT2 expression in limb muscles of COPD patients is likely to originate from within muscle cells themselves. Our group, as well as that of Dallabrida *et al*. [8,10], have suggested that human and murine skeletal cells do indeed produce ANGPT2. To evaluate this possibility, primary human and murine skeletal myoblasts were isolated, purified and assessed for ANGPT2 production during muscle differentiation. Our results clearly illustrate that ANGPT2 is produced by skeletal myoblasts and that ANGPT2 production increases substantially upon differentiation into myotubes (Figure 2). Another cell type that may contribute to elevated ANGPT2 expression in limb muscles of COPD patients is endothelial cells. Endothelial cells are well-known to produce higher levels of ANGPT2 than do other cells, and are primarily regulated by transcriptional activation of ANGPT2 promoter [12,33], but

also due to the fact that ANGPT2 release can be rapidly achieved from stores inside Weibel-Palade bodies [34]. The relative contribution of endothelial vs. skeletal muscle cells to overall ANGPT2 skeletal muscle production remains to be determined.

Another important finding in our study is that ANGPT2 expression in the *vastus lateralis* correlates positively with muscle TNFα expression (Figure 1). A similar linear correlation has been described between plasma ANGPT2 and TNFα concentrations in humans with endotoxemia and sepsis [14,15]. Our finding implies that enhanced ANGPT2 production in limb muscles of COPD patients is mediated by a direct effect of TNFα on muscle cells and possibly on endothelial cells. Regulation of ANGPT2 expression by TNFα has been studied in endothelial cells with contradictory results. An initial report by Kim *et al*. [35] described significant but transient upregulation of ANGPT2 expression in HUVECs exposed to TNFα. Subsequent studies [34,36], however, failed to show any significant effect of TNFα on endothelial ANGPT2 expression or release. To verify the direct influence of TNFα on cellular ANGPT2 expression, we exposed both, human skeletal myoblasts and endothelial cells to TNFα for 24h and found no significant changes in ANGPT2 expression in these cells (Figure 2). These results suggest that TNFα is unlikely to be the factor responsible for enhanced ANGPT2 expression in limb muscles of COPD patients and that, more likely, both ANGPT2 and TNFα are regulated by a common mediator. One such mediator is oxidative stress. Numerous reports have confirmed that both indirect and direct measures of oxidative stress are elevated in the serum and limb muscles of patients with COPD (for review, please see [37]). Our study indicates that H_2O_2 elicits significant inductions of ANGPT2

production both in human skeletal myoblasts and in endothelial cells suggesting that both cell types can contribute to enhanced ANGPT2 production in response to oxidative stress (Figure 2). These results are in agreement with those of Amano *et al*., who reported elevated ANGPT2 expression in pericytes exposed to H_2O_2 [38]. The mechanisms, however, through which H_2O_2 induce ANGPT2 expression in skeletal muscles remain speculative. We speculate that H_2O_2 may activate ETS transcription factors [39] and that these factors, due to the abundance of their binding sites on human ANGPT2 promoter, are likely responsible for ANGPT2 production in both muscle and endothelial cells in response to oxidative stress [33].

3.5.3 ANGPT2 REGULATION OF MYOGENESIS

The only available information regarding the influence of ANGPT2 on skeletal muscle is that of Dallabrida *et al.* [8], who reported that cultured skeletal satellite cells adhere to ANGPT1 and ANGPT2 proteins; however, whereas ANGPT1 promotes survival and inhibits apoptosis, ANGPT2 enhances apoptosis. We report here for the first time, that while ANGPT2 has no effect on proliferation and migration of skeletal myoblasts, it strongly promotes myoblast survival and inhibits apoptosis (Figure 3). ANGPT2 also strongly enhances differentiation of myoblasts into myotubes, as evidenced by substantial induction of muscle-specific transcription factors (MyoD and myogenin) and muscle-specific proteins (creatine kinase and myosin heavy chain) (Figure 5).

Little is as yet known about the ability of primary cells to repair muscle fiber injury in skeletal muscles of COPD patients. Martinez-Llorens *et al*. [17] have reported that satellite cell number and activation status increase significantly in the external intercostal muscles of COPD patients, as compared to control subjects,

indicating the presence of an active muscle fiber repair program. Our findings, that ANGPT2 enhances both differentiation and survival of myoblasts, strongly suggest that ANGPT2 plays a role in the muscle fiber repair process, since augmented ANGPT2 expression in COPD patients may represent a positive physiological adaptation - one that is designed to enhance the regenerative capacities of the muscles in response to injury.

Differences between our conclusions and those of Dallabrida *et al*. regarding the influence of ANGPT2 on skeletal myoblast survival may be attributed to methodological differences. Dallabrida and colleagues focused their attention on measuring adhesion of myoblasts to extracellular matrices composed of ANGPT1, ANGPT2 or other proteins such as collagens, fibronectin, vitronectin and laminin. Their approach was based on an earlier report indicating that ANGPT1 is incorporated into extracellular matrices of cells [40]. However, it remains unknown whether or not ANGPT2 protein is actually incorporated into the extracellular matrices of skeletal myoblasts, so we assessed cell survival using a physiological approach that exposed skeletal myoblasts to ANGPT2 protein over 36h.

3.5.4 MECHANISMS OF ANGPT2 ACTION IN MYOBLASTS

We used two approaches to evaluate signaling mechanisms through which ANGPT2 regulates skeletal myoblast survival and differentiation. First, we measured activation of the PI3 kinase/AKT and ERK1/2 pathways in myoblasts exposed to ANGPT2. We found that ANGPT2 transiently and dose-dependently activates AKT and ERK1/2 phosphorylation and that ANGPT2 has no effect on starvation-induced cytotoxicity (index of overall cell death) and caspase 3/7

activation (index of apoptotic cell death) in the presence of selective inhibitors of PI3 kinase, AKT and ERK1/2. These results suggest that the PI3 kinase and ERK1/2 pathways are critical to the pro-survival and anti-apoptotic effects of ANGPT2 in skeletal muscle cells. This conclusion mirrors what has already been established in endothelial cells [29], although it is unclear in the case of skeletal myoblasts whether ANGPT2 pathway activation is mediated through phosphorylation of TIE2 receptors, ligation of integrins or both.

The second approach that we used to gain further insight into the mechanisms through which ANGPT2 modulates skeletal myoblast differentiation, survival and apoptosis was to compare the transcriptomes of myoblasts expressing GFP (control) and those overexpressing ANGPT2. Our results indicate that ANGPT2 triggers specific changes in the muscle transcriptome that involve the upregulation of several genes connected to cell signaling, including the regulation of cell cycle, assembly and organization, cell growth and proliferation. The protein that is most strongly-induced by ANGPT2 is NRP1 (neuropilin 1), which is a transmembrane receptor for semaphorins (mediators of neuronal guidance) and VEGFA [41]. NRP1 is expressed in skeletal muscle progenitor cells (myoblasts) and its expression is augmented during differentiation into myotubes [41]. These observations, along with the fact that VEGF strongly promotes skeletal myoblast survival and enhances myogenic differentiation, suggest that ANGPT2 may regulate myogenesis through augmentation of VEGF signaling. The protein that is second most induced by ANGPT2 is telomere maintenance 2 (TELO2), which functions as an S-phase checkpoint protein in the cell cycle and is a key element in embryonic development [42].

ANGPT2 also significantly induces the expression and release of leptin from myoblasts, suggesting that the pro-myogenic and pro-survival effects of ANGPT2 are likely mediated by leptin (figure 6). Leptin was identified initially as an adipocyte-derived hormone that regulates food intake through activation of receptors located in the hypothalamus. However, leptin is now recognized as a multipotent cytokine with numerous central and peripheral effects. Leptin and leptin receptor mRNA and proteins have been detected in mature skeletal muscle fibers and progenitor cells [43]. In muscle cells, leptin strongly influences metabolism by promoting both, free fatty acid oxidation and glucose uptake [44]. It also protects muscle cells from oxidative stress-induced apoptosis and prevents protein breakdown in C2C12 cultured myotubes [45,46]. These observations suggest that the influence of ANGPT2 on skeletal muscle survival may be mediated through secondary release of leptin which can act on muscle cells through autocrine mechanism.

In addition to the above-described modulators of cell cycle and metabolism, network analysis of ANGPT2-regulated genes in muscle cells revealed that ANGPT2 downregulate many pro-inflammatory mediators including the inducible nitric oxide synthase, IL6 and members of cellular networks involving IFNα, IL1, IL12 and AP-1 and NFκB transcription factors (Figure 1 and supplementary figure 1). These cytokines and transcription factors are well known positive modulators of catabolism and muscle wasting. On the basis of these results, we speculate that ANGPT2 promotes muscle cells survival and differentiation through upregulation

of key regulators of cell cycle, protein synthesis and metabolism and inhibition of the expression of mediators of protein degradation and catabolism.

In summary, our study indicates that ANGPT2 expression is significantly elevated in the *vastus lateralis* of COPD patients and that skeletal muscle ANGPT2 expression is positively regulated by H_2O_2. We also found that ANGPT2 triggers significant increases in survival and differentiation of skeletal myoblasts and that these effects are mediated through coordinated regulation of several modulators of cell survival, metabolism, protein synthesis and degradation.

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3.7 AUTHORS CONTRIBUTIONS:

Conceived and designed the experiments: MM SH
Performed the experiments: MM FM DM
Analyzed the data: MM DM
Contributed reagents/materials: FM
Wrote the paper: MM SH

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42. Takai H, Wang RC, Takai KK, Yang H, de LT (2007) Tel2 regulates the stability of PI3K-related protein kinases. Cell 131: 1248-1259.

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44. Suzuki A, Okamoto S, Lee S, Saito K, Shiuchi T, Minokoshi Y (2007) Leptin stimulates fatty acid oxidation and peroxisome proliferator-activated receptor alpha gene expression in mouse C2C12 myoblasts by changing the subcellular localization of the alpha2 form of AMP-activated protein kinase. Mol Cell Biol 27: 4317-4327.

45. Ramsay TG (2003) Porcine leptin inhibits protein breakdown and stimulates fatty acid oxidation in C2C12 myotubes. J Anim Sci 81: 3046-3051.

46. Eguchi M, Liu Y, Shin EJ, Sweeney G (2008) Leptin protects H9c2 rat cardiomyocytes from H2O2-induced apoptosis. FEBS J 275: 3136-3144.

3.9 FIGURES

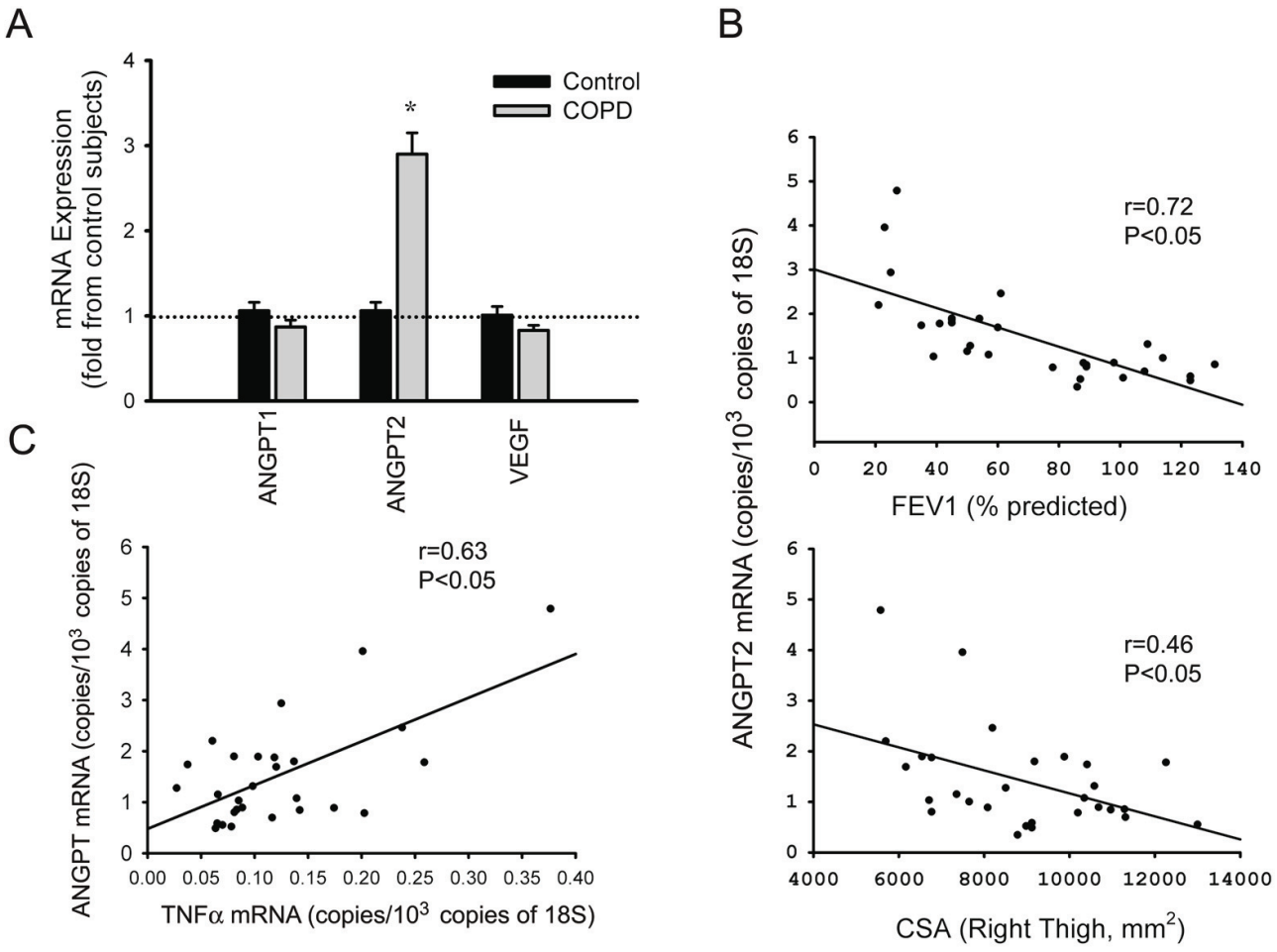


FIGURE 1

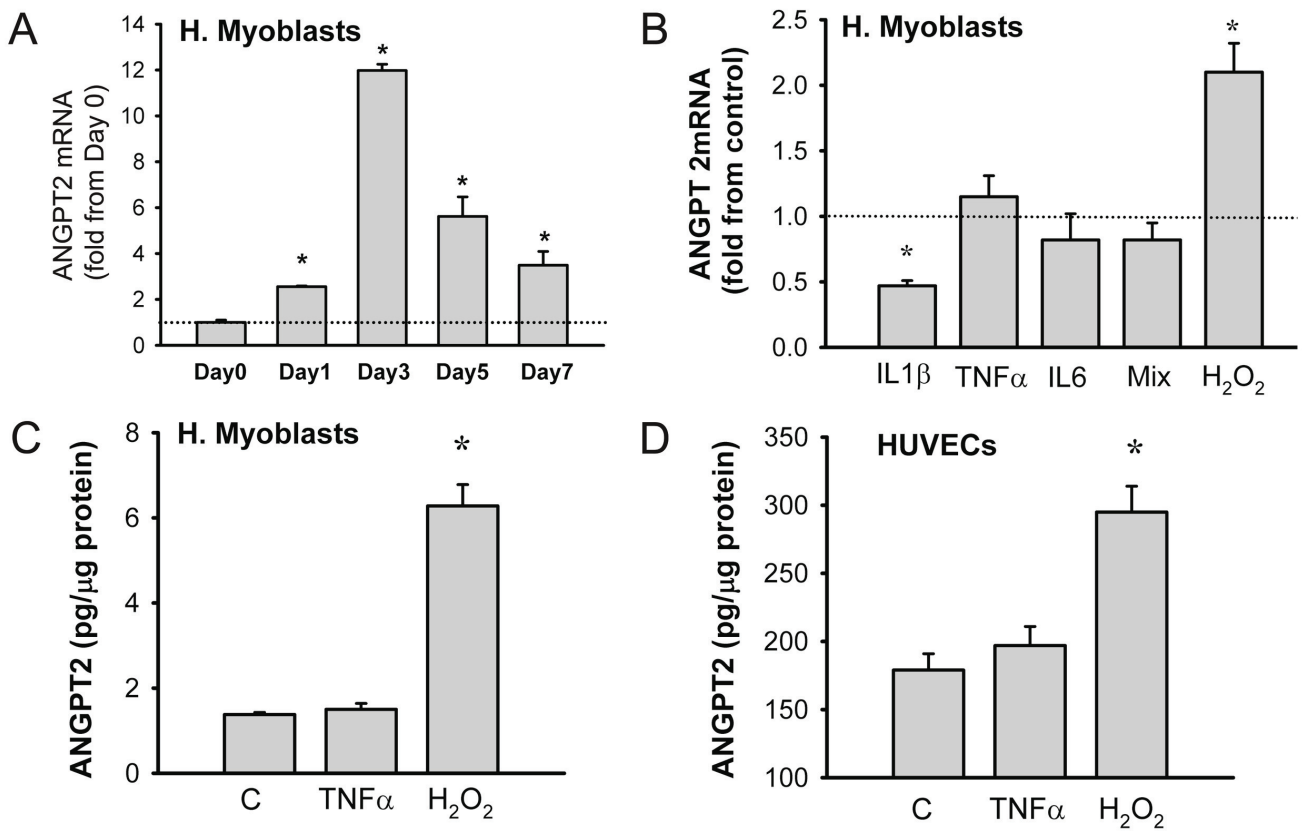

A
H. Myoblasts
ANGPT2 mRNA (fold from Day 0)
Day0
Day1
Day3
Day5
Day7
B
H. Myoblasts
ANGPT 2mRNA (fold from control)
IL1β
TNFα
IL6
Mix
H2O2
C
H. Myoblasts
ANGPT2 (pg/μg protein)
C
TNFα
H2O2
D
HUVECs
ANGPT2 (pg/μg protein)
C
TNFα
H2O2

FIGURE 2

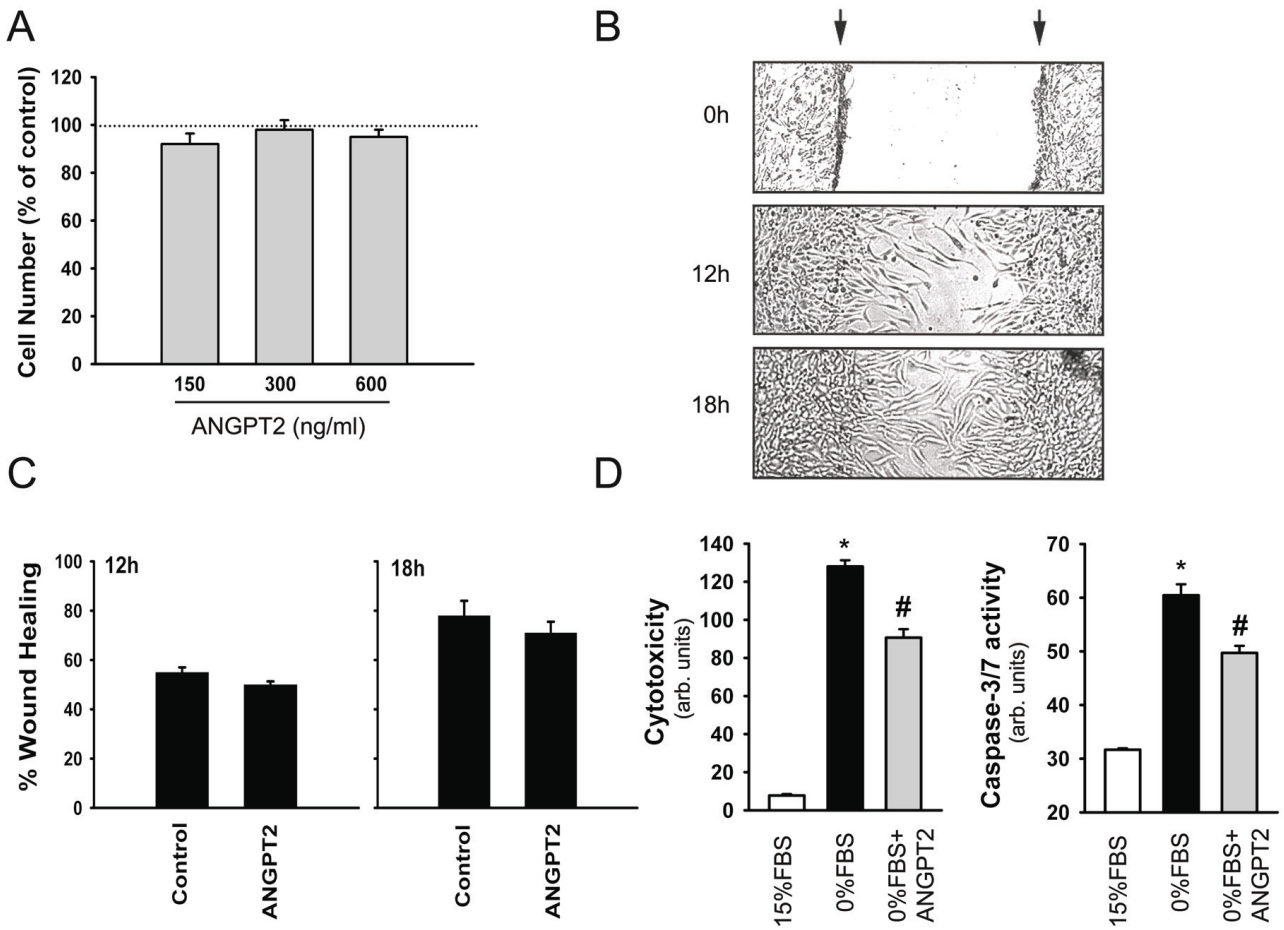

A
Cell Number (% of control)
120
100
80
60
40
20
0
150
300
600
ANGPT2 (ng/ml)
B
0h
12h
18h
C
% Wound Healing
12h
18h
100
80
60
40
20
0
Control
ANGPT2
Control
ANGPT2
D
Cytotoxicity
(arb. units)
140
120
100
80
60
40
20
0
*
#
15%FBS
0%FBS
0%FBS+ ANGPT2
Caspase-3/7 activity
(arb. units)
70
60
50
40
30
20
*
#
15%FBS
0%FBS
0%FBS+ ANGPT2

FIGURE 3

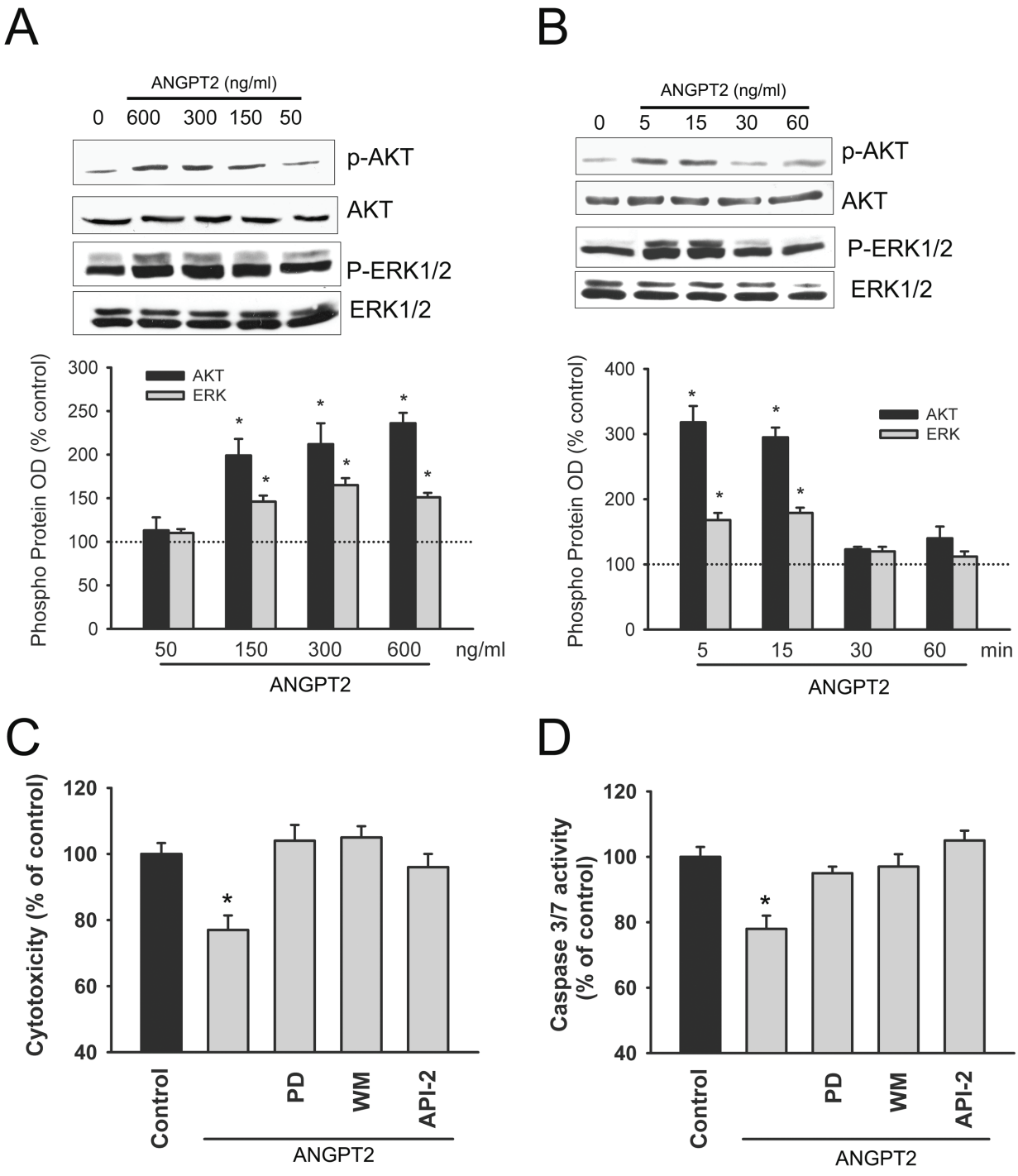

A
ANGPT2 (ng/ml)
0 600 300 150 50
p-AKT
AKT
P-ERK1/2
ERK1/2
Phospho Protein OD (% control)
AKT
ERK
50 150 300 600 ng/ml
ANGPT2
B
ANGPT2 (ng/ml)
0 5 15 30 60
p-AKT
AKT
P-ERK1/2
ERK1/2
Phospho Protein OD (% control)
AKT
ERK
5 15 30 60 min
ANGPT2
C
Cytotoxicity (% of control)
Control
PD
WM
API-2
ANGPT2
D
Caspase 3/7 activity (% of control)
Control
PD
WM
API-2
ANGPT2

FIGURE 4

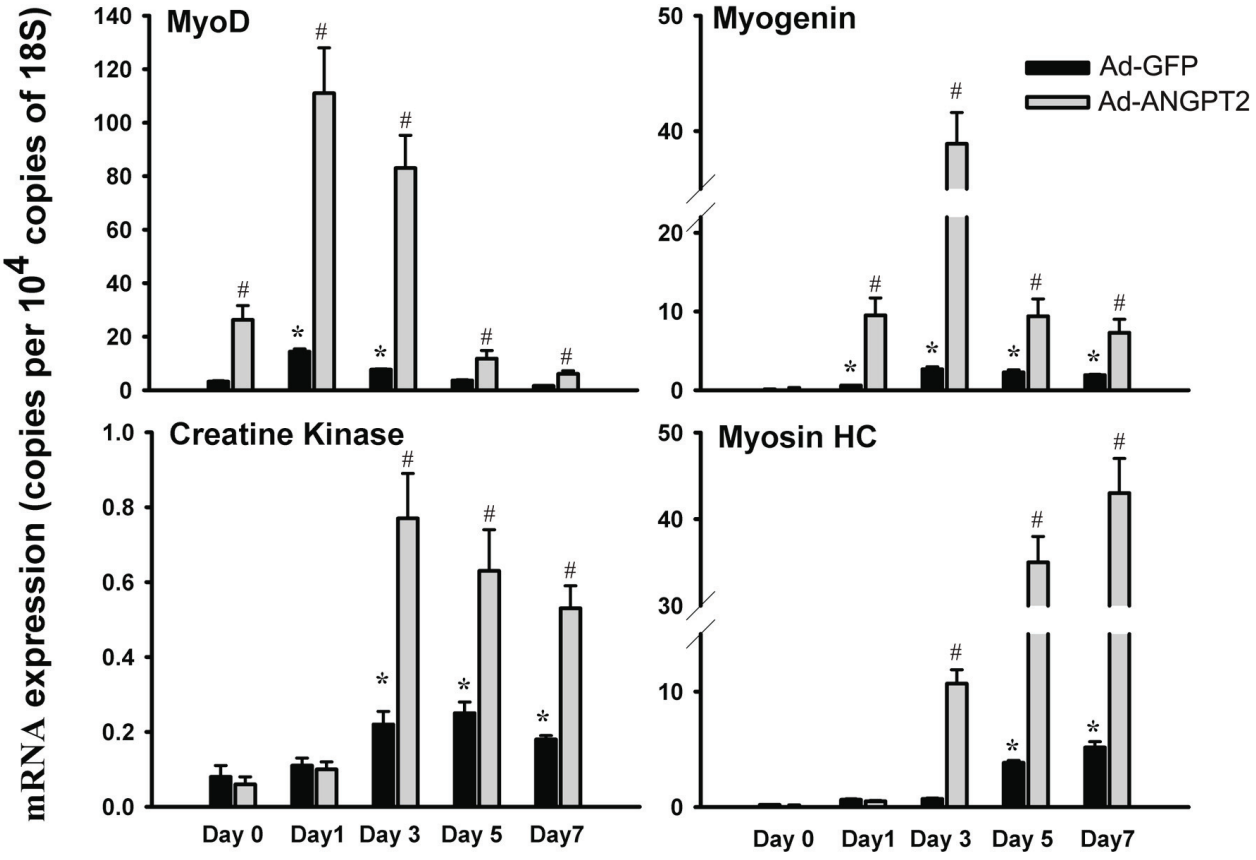

MyoD
Myogenin
Creatine Kinase
Myosin HC
Ad-GFP
Ad-ANGPT2
mRNA expression (copies per 10^4 copies of 18S)
Day 0
Day1
Day 3
Day 5
Day7

FIGURE 5

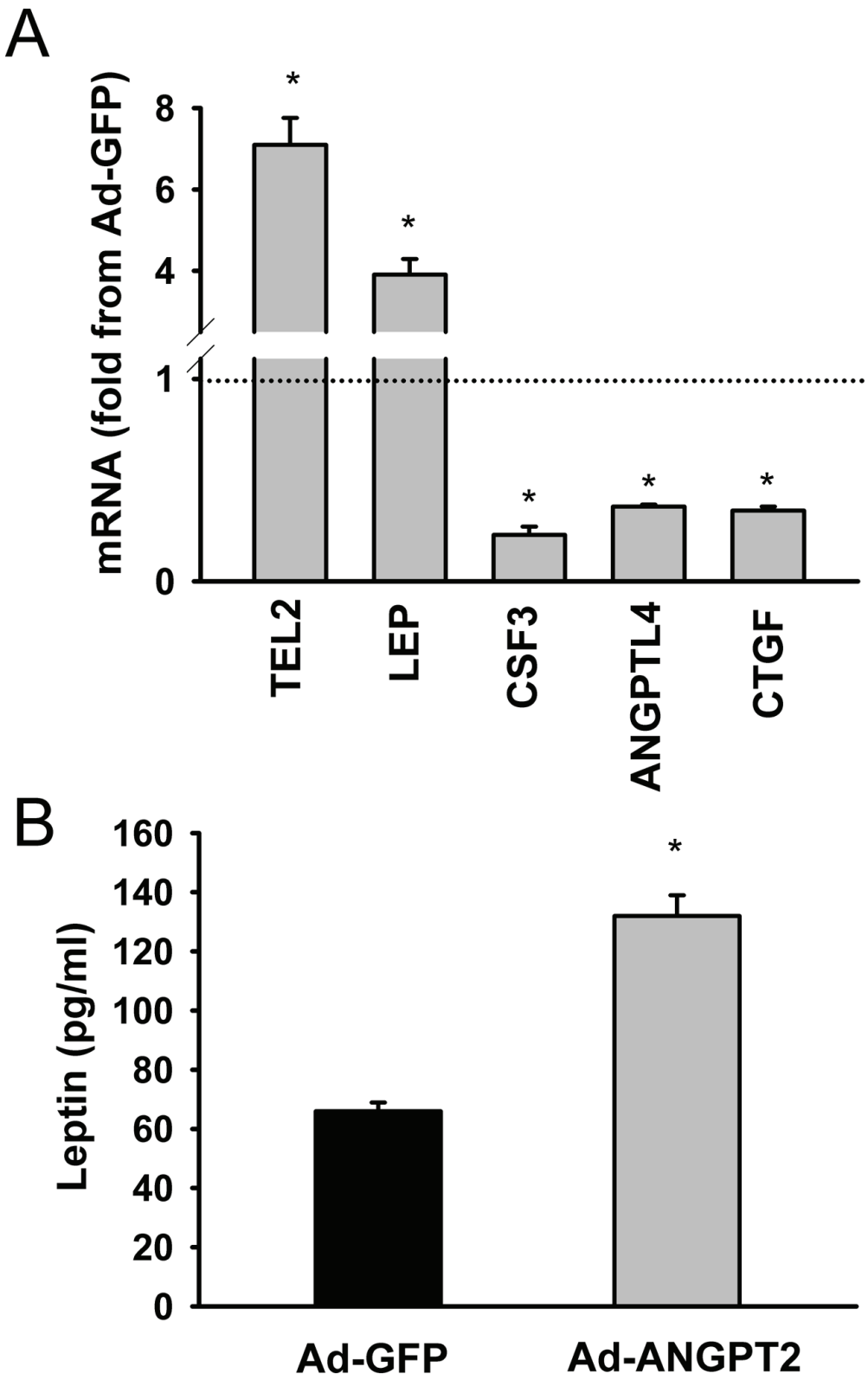

A
mRNA (fold from Ad-GFP)
8
6
4
1
0
*
*
*
*
*
TEL2
LEP
CSF3
ANGPTL4
CTGF
B
Leptin (pg/ml)
160
140
120
100
80
60
40
20
0
*
Ad-GFP
Ad-ANGPT2

FIGURE 6

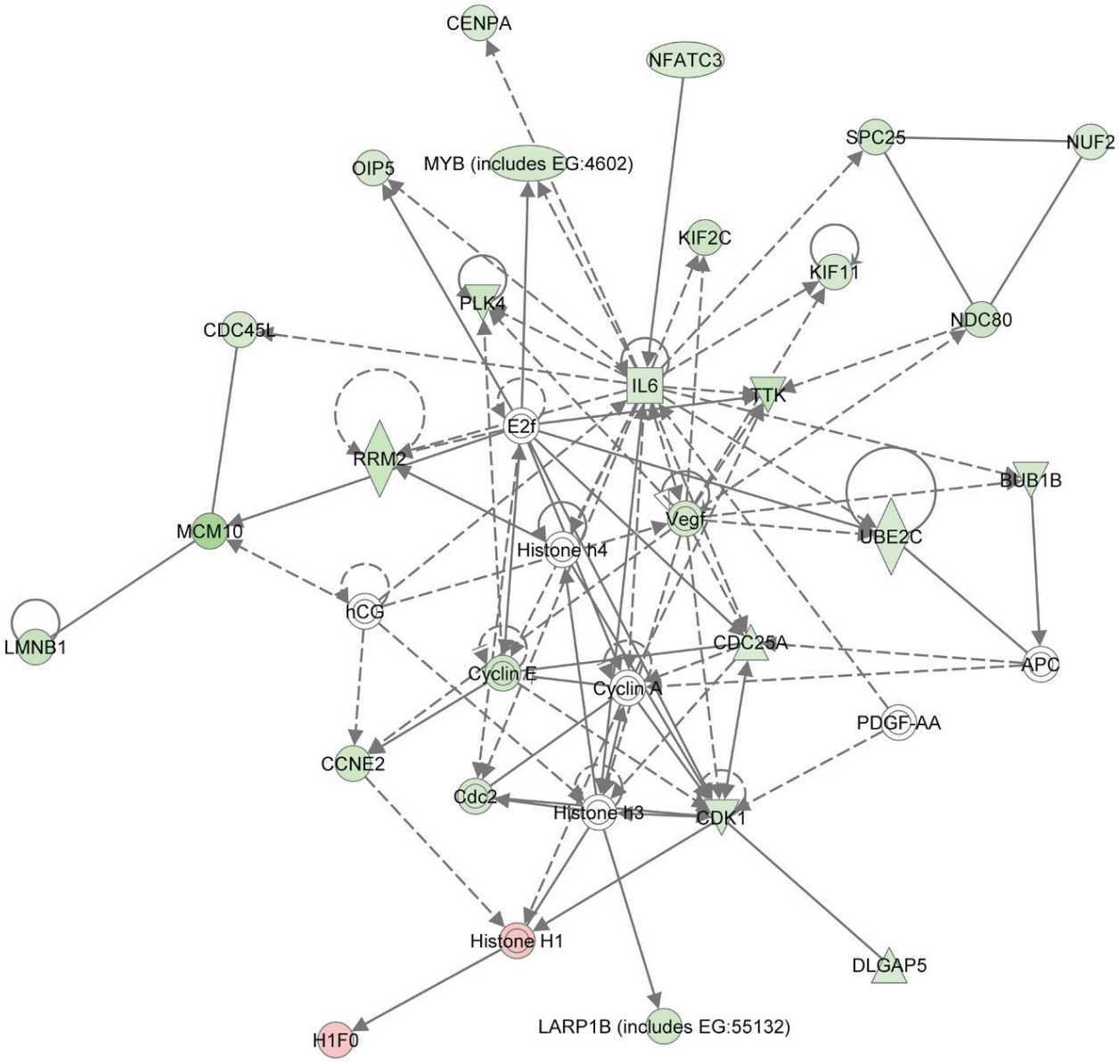

CENPA
NFATC3
SPC25
NUF2
OIP5
MYB (includes EG:4602)
KIF2C
KIF11
PLK4
NDC80
CDC45L
IL6
TTK
E2f
RRM2
BUB1B
Vegf
MCM10
UBE2C
Histone h4
hCG
LMNB1
CDC25A
APC
Cyclin E
Cyclin A
PDGF-AA
CCNE2
Cdc2
Histone h3
CDK1
Histone H1
DLGAP5
H1F0
LARP1B (includes EG:55132)

FIGURE 7

3.10 FIGURE LEGENDS:

Figure 1:
A: mRNA expressions of ANGPT1, ANGPT2 and VEGF in *vastus lateralis* muscles of control subjects and COPD patients (Study #2). *P<0.05 compared with control.
B-C: Relationships between mRNA expressions of ANGPT2 and FEV_1, right thigh cross sectional areas (CSA), and TNFα mRNA expression in *vastus lateralis* muscle of control subjects and COPD patients.

Figure 2:
A: mRNA expression of ANGPT2 in human skeletal myoblasts (day 0) and after 1, 3, 5 and 7 days of differentiation into myotubes. Values are means ± SE. *P<0.05 compared with day 0.
B: ANGPT2 mRNA expression in human skeletal myoblasts exposed for 24h to IL1β, TNFα, IL6, a combination of the three, or H_2O_2. N=6. *P<0.05 compared with control.
C: ANGPT2 protein levels in the media of human skeletal myoblasts exposed for 24h to BSA (control, C), TNFα or H_2O_2. N=6. *P<0.05 compared with control.
D: ANGPT2 protein levels in the media of human umbilical vein endothelial cells (HUVECs) exposed for 24h to PBS (control, C), TNFα or H_2O_2. N=6. *P<0.05 compared with control.

Figure 3:
A: Cell number of human skeletal myoblasts exposed to PBS (control) or ANGPT2. Cells maintained for 72 in culture medium. N=6.
B: Representative Photographs of wound healing assays in human skeletal myoblasts. Arrows indicate margins of wounds.
C: Wound healing intensity in myoblasts exposed to PBS (control) or ANGPT2. N=6 per group.
D: Cytotoxicity and caspase 3/7 activity in human skeletal myoblasts maintained for 36h in media containing 15%FBS, 0%FBS or 0%FBS + ANGPT2. N=8 per group. *P<0.05 compared with 15% FBS, $^{\#}$P<0.05 compared with 0% FBS.

Figure 4:
A: Representative immunoblots and optical densities of total and phosphorylated AKT and ERK1/2 proteins in human skeletal myoblasts exposed for 15 min to increasing concentrations of ANGPT2. N=4 per group.
B: Representative immunoblots and optical densities of total and phosphorylated AKT and ERK1/2 proteins in human skeletal myoblasts exposed to 600 ng/ml ANGPT2 for increasing durations. N=4 per group.
C-D: Cytotoxicity and caspase 3/7 activity in human skeletal myoblasts maintained for 36h in media containing 0%FBS (control) or 0%FBS + ANGPT2 in the presence of PD184352, (PD, ERK1/2 inhibitor), wortmannin, (WM, PI3 kinase inhibitor) and API2 (AKT inhibitor). *P<0.05 compared with control.

Figure 5: mRNA expressions of MyoD, myogenin, creatine kinase and myosin heavy chain in human skeletal myoblasts infected with Ad-GFP (control) or Ad-ANGPT2 viruses. Cells were collected at the myoblast phase (day 0) and after 1, 3, 5 and 7 days of differentiation into myotubes. N=6 per group. *P<0.05 compared with day 0 (myoblast phase). # P<0.05 compared with cells infected with Ad-GFP.

Figure 6:
A: Expressions of TELO2, LEP, CSF3 and ANGPTL4 in human skeletal myoblasts infected with Ad-ANGPT2 viruses. Values are expressed as fold changes from those measured in cells infected with Ad-GFP (control).
B: Expression of leptin in human skeletal myoblasts infected with Ad-GFP (control) and Ad-ANGPT2 viruses.
*P<0.05 compared with Ad-GFP.

Figure 7: The top network of regulated genes in human myoblasts infected with Ad-ANGPT2 viruses vs. cells infected with Ad-GFP viruses (control condition). This figure was created with the Ingenuity Pathway Analysis system. Upregulated genes are shown in red while downregulated genes are shown in green. Color intensity indicates the degree of change in gene expression. White nodes are genes whose expression was not significantly changed in cells infected with Ad-ANGPT2 viruses vs. Ad-GFP viruses. Arrows with plain lines indicate direction interactions, and arrows with interrupted lines represent indirect interactions. Top functions associated with this network include cell cycle, cellular assembly and organization, DNA replication, recombination and repair.

3.11 TABLE 1: Characteristics of control subjects and COPD patients who underwent *vastus lateralis* biopsies.

	Control (n=14)	**COPD** (n=16)
Age, yr	65.4±2.1	71.2±3.3
Male, %	100	100
Height, m	1.70±0.02	1.69±0.02
Weight, kg	79.4±2.7	75.8±3.4
BMI, kg/m^2	27.4±0.9	26.4±1.1
Left Thigh CSA, mm^2	9662±478	7765±387*
FEV_1, l	3.15±0.21	1.24±0.09*
FEV_1, % predicted	101.7±4.5	42.4±3.1*
FVC, l	4.05±0.23	2.73±0.14*
FVC, % predicted	104.2±4.2	72.13±3.1*
FEV_1/FVC, %	77.6±1.7	45.9±1.8*
Muscle TNFα mRNA Copies/10^3 copies of 18S	0.105±0.011	0.413±0.021*
Muscle IL1β mRNA Copies/10^3 copies of 18S	3.93±1.40	3.10±1.10
Muscle IL6 mRNA Copies/10^3 copies of 18S	0.038±0.01	0.046±0.01

BMI: Body mass index; CSA: cross sectional area. Data are presented as mean ± SE. * $p<0.001$ compared with control subjects.

3.12 TABLE 2: Summary of genes whose expression is significantly altered in Ad-ANGPT2-infected myoblasts as compared to cells infected with Ad-GFP viruses.

Genes induced by ANGPT2 in skeletal myoblasts

Accession	Symbol	Definition	Ad-ANGPT2/ Ad-GFP
	Function		
NM001024629	NRP1	Neuropilin1 transcript variant 3	12.9
	Angiogenesis		
NM016111	TELO2	Telomere maintenance 2 homology	12.0
	Cell cycle		
NM201414	APP	Amyloid beta A4 precursor	8.9
	Apoptosis		
NM033397	ITPRIP	Inositol triphosphate interacting protein	3.2
	Signaling		
NM032340	C6orf125	Chromosome 6 open reading frame125	3.1
	Carbohydrase transport		
NM000230	LEP	Leptin	3.1
	Signaling		
NM182480	COQ6	Coenzyme Q6 homolog	2.9
	Electron transport		
NM181877	ZSCAN2	Zinc finger and SCAN domain containing 2	2.8
	Transcription		
NM032645	C10orf33	Chromosome 10 open reading frame 33	2.7
	Electron transport		
NM032645	RAPSN	Receptor-associated protein of synapse	2.6
	Proteolysis		
NM016401	C11orf73	Chromosome 11 open reading frame 73	2.6
	Unknown		
NM001011539	LOC44104	Hypothetical LOC441046	2.5
	Metabolism		
NM199295	APITD1	Apoptosis-induced TAF9-like domain 1	2.5
	Transcription		

Genes inhibited by ANGPT2 in skeletal myoblasts

NM002155	HSPA6	Heat shock 70 kDa protein 6	0.03
	Protein folding		
NM178539	FAM19A2	Family with sequence similarity 19	0.05
	Signaling		
NM001012977	PAP1M	Polyadenylate-binding protein 1	0.05
	RNA stability		
NM005345	HSPA1A	Heat shock 70kDa protein 1A	0.16
	Protein folding		
NM139016	C20orf198	Chromosome 20 open reading frame 198	0.20
	Unknown		
NM005346	HSPA1B	Heat shock 70kDa protein	0.21
	Protein folding		
NM001002796	MCTP1	Multiple C2 domains, transmembrane 1	0.21
	Ca++-mediated signaling		
NM005252	FOS	FBJ murine osteosarcome oncogene homolog	0.22
	Transcription		

NM032461	SPANXB1	Spanx family, member B1	0.26
	Transcription		
NM153292	NOS2A	Nitric oxide synthase 2A	0.26
	Signaling		
NM030926	ITM2C	Integral membrane protein 2C	0.26
	Unknown		
NM013453	SPANXA1	Spanx family member A1	0.27
	Transcription		
NM000488	SERPINC1	Serine peptidase inhibitor, clade C	0.27
Apoptosis			
NM172220	CSF3	Colony stimulating factor 3	0.28
	Immune responses		
NM018602	DNAJA4	DNAJ (Hsp40) homolog	0.28
	Protein folding		
NM022661	SPANXC	Spanx family member C	0.28
	Transcription		
NM001005611	EDA	Ectodysplasin A	0.29
	Immune responses		

3.13 TABLE 3: Summary of the results of Ingenuity Pathway Analysis of lists of upregulated and downregulated genes in human skeletal myoblasts infected with Ad-Ang-2 compared with cells infected with Ad-GFP (control):

Top Networks

Associated Network Functions	Score	Focus Molecules
Cell cycle, DNA replication, Recombination and Repaid	50	24
Tissue Morphology, Lipid Metabolism, Small Molecule Biochemistry	35	18
Cardiovascular System Development and Function, Tissue Development	25	14
Cellular Assembly and Organization	19	11
Cell Morphology, Cellular Development	16	10
Connective Tissue Development and Function	15	10
Cellular Comprise	14	9

Molecular and Cellular Functions

Name	# Molecules
Cell Cycle	32
Cell Death	42
Cellular Assembly and Organization	25
DNA Replication, Recombination and Repair	29
Cellular Growth and Proliferation	37

3.14 ON LINE DATA SUPPLEMENT

Table S1: Primers used for real-time PCR experiments to detect the expression of MyoD, myogenin, myosin heavy chain (MyoHC) and creatine kinase (CK) during differentiation of human skeletal myoblasts into myotubes.

Gene			Accession #
MyoD	Forward	5'- TCTCTGCTCCTTTGCCACAA -3'	NM_002478
	Reverse	5'- AGTGCTCTTCGGGTTTCAGG -3'	
Myogenin	Forward	5'- GAAGGTGAATGAGGCCTTCG -3'	NM_002479
	Reverse	5'- AGGCGCTCGATGTACTGGAT -3'	
MyoHC, fast	Forward	5'- AGCTGGACGAAAGGCTCAAG -3'	NM_002470
	Reverse	5'- CATAGTCGCTGCGCTGTTTC -3'	
CK, muscle	Forward	5'- AGTACCCCGACCTCAGCAAA -3'	NM_001824
	Reverse	5'- GGTGACCTGGGTTGTCCACT -3'	

Table S2: List of genes whose expression is significantly upregulated in skeletal myobalsts infected with Ad-Ang-2 compared with cells infected with Ad-GFP.

Accession	Definition		fold change (log2)	P-value
NM_001024629	NRP1	neuropilin 1 (NRP1), transcript variant 3	3.6	0.000001
NM_016111	TELO2	Telomere maintenance 2 homology	3.5	0.000002
NM_201414	APP	amyloid beta (A4) precursor protein (APP), transcript variant 3	3.1	0.000003
NM_033397	ITPRIP	Inositol 1,4,5-triphosphate interacting protein	1.6	0.000001
NM_032340	C6orf125	chromosome 6 open reading frame 125	1.6	0.000001
NM_0002301	LEP	leptin (obesity homolog, mouse)	1.6	0.000094
NM_182480	COQ6	coenzyme Q6 homolog, monooxygenase	1.5	0.000007
NM_181877	ZSCAN2	zinc finger and SCAN domain containing 2 transcript variant 1.	1.5	0.000003
NM_032709	C10orf33	chromosome 10 open reading frame 33	1.5	0.000006
NM_032645	RAPSN	receptor-associated protein of the synapse, transcript variant 2	1.4	0.000012
NM_016401	HSPC138	hypothetical protein HSPC138	1.3	0.000002
NM_001011539	LOC441046	hypothetical LOC 441046	1.3	0.001073
NM_199295	APITD1	apoptosis-inducing, TAF9-like domain 1, transcript variant B	1.3	0.000033
NM_054012	ASS	argininosuccinate synthetase (ASS), transcript variant 2	1.3	0.002185
NM_138334	JOSD2	Josephin domain containing 2	1.3	0.000053
NM_000266	NDP	Norrie disease (pseudoglioma)	1.2	0.000114
NM_001014999	GIYD1	GIY-YIG domain containing 1 (GIYD1), transcript variant 1	1.2	0.005307
NM_001012716	C18orf56	chromosome 18 open reading frame 56	1.2	0.000017
NM_005318	H1F0	H1 histone family, member 0	1.1	0.000040
NM_145168	HSPC105	NAD(P) dependent steroid dehydrogenase-like	1.1	0.008313
NM_014370	STK23	serine/threonine kinase 23	1.1	0.008618
NM_001017915	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa transcript variant 1	1.1	0.000651
NM_030590	MATN4	matrilin 4 (MATN4), transcript variant 2	1.1	0.004696
NM_005954	MT3	metallothionein 3 (growth inhibitory factor (neurotrophic))	1.1	0.004652
NM_139125	MASP1	mannan-binding lectin serine peptidase 1 (MASP1), transcript variant 2	1.1	0.001030
NM_207510	FLJ45224	FLJ45224 protein	1.1	0.003391

NM_052859	RFT1	RFT1 homolog (S. cerevisiae)	1.1	0.001279
NM_004704	RNU3IP2	RNA, U3 small nucleolar interacting protein 2	1.1	0.000008
NM_016246	DHRS10	dehydrogenase/reductase (SDR family) member 10	1.1	0.000008
NM_001647	APOD	apolipoprotein D	1.1	0.000062
NM_002978	SCNN1D	sodium channel, nonvoltage-gated 1, delta	1.1	0.006010
NM_000394	CRYAA	crystallin, alpha A	1.1	0.021343
NM_001003795	GTF2IRD2B	GTF2IRD2 beta.	1.1	0.000486
NM_203296	TRIM7	tripartite motif-containing 7 (TRIM7), transcript variant 3	1.1	0.016131
NM_000422	KRT17	keratin 17	1.0	0.000348
NM_138355	SCRN2	secernin 2	1.0	0.001370
NM_152472	ZNF578	zinc finger protein 578	1.0	0.002666
NM_006948	STCH	stress 70 protein chaperone, microsome-associated, 60kDa	1.0	0.000365
NM_173666	DTWD2	DTW domain containing 2	1.0	0.003105
NM_001001891	TMEM16G	transmembrane protein 16G, transcript variant NGEP-L	1.0	0.018691
NM_080748	C20orf52	chromosome 20 open reading frame 52	1.0	0.000029
NM_182970	RIMS4	regulating synaptic membrane exocytosis 4	1.0	0.004992
NM_052880	MGC17330	HGFL gene	1.0	0.000039
NM_021182	HB-1	minor histocompatibility antigen HB-	1.0	0.001319
NM_006983	MMP23B	matrix metallopeptidase 23B	1.0	0.006271

Table S3: List of genes whose expression is significantly downregulated in skeletal myobalsts infected with Ad-Ang-2 compared with cells infected with Ad-GFP.

Accession		Definition	Fold change	P-value (log2)
NM_031423	CDCA1	cell division cycle associated transcript variant 2	-1.01	0.00021
NM_018410	DKFZ	hypothetical protein DKFZ (DKFZp762E1312)	-1.01	0.00089
NM_152326	ANKRD9	ankyrin repeat domain 9	-1.01	0.00669
NM_018193	KIAA1794	KIAA1794	-1.02	0.00025
NM_014143	CD274	CD274 antigen	-1.02	0.00299
NM_001789	CDC25A	cell division cycle 25A transcript variant 1	-1.02	0.00002
NM_001067	TOP2A	topoisomerase (DNA) II alpha 170kDa	-1.02	0.00007
NM_173163	NFATC3	nuclear factor of activated T-cells transcript variant 3	-1.03	0.00452
NM_001010980	C1orf130	chromosome 1 open reading frame 130	-1.04	0.00001
NM_001005158	SFMBT1	Scm-like with four mbt domains 1, transcript variant 2	-1.04	0.01017
NM_018478	C20orf35	chromosome 20 open reading frame 35 transcript variant 1	-1.04	0.00001
NM_001809	CENPA	centromere protein A, 17kDa	-1.05	0.00039
NM_199420	POLQ	polymerase (DNA directed), theta	-1.05	0.00843
NM_006733	FSHPRH1	FSH primary response (LRPR1 homolog, rat) 1	-1.05	0.00172
NM_017779	DEPDC1	DEP domain containing 1	-1.06	0.00074
NM_152515	FLJ40629	hypothetical protein FLJ40629	-1.06	0.00006
NM_014750	DLG7	discs, large homolog 7 (Drosophila)	-1.06	0.00009
NM_080668	CDCA5	cell division cycle associated 5	-1.06	0.00002
NM_000600	IL6	interleukin 6 (interferon, beta 2)	-1.06	0.00122
NM_194293	CMYA1	cardiomyopathy associated 1	-1.06	0.00361
NM_001907	CTRL	chymotrypsin-like	-1.07	0.00189
NM_000057	BLM	Bloom syndrome	-1.07	0.00789
NM_1768701	MT1M	metallothionein 1M	-1.07	0.00986

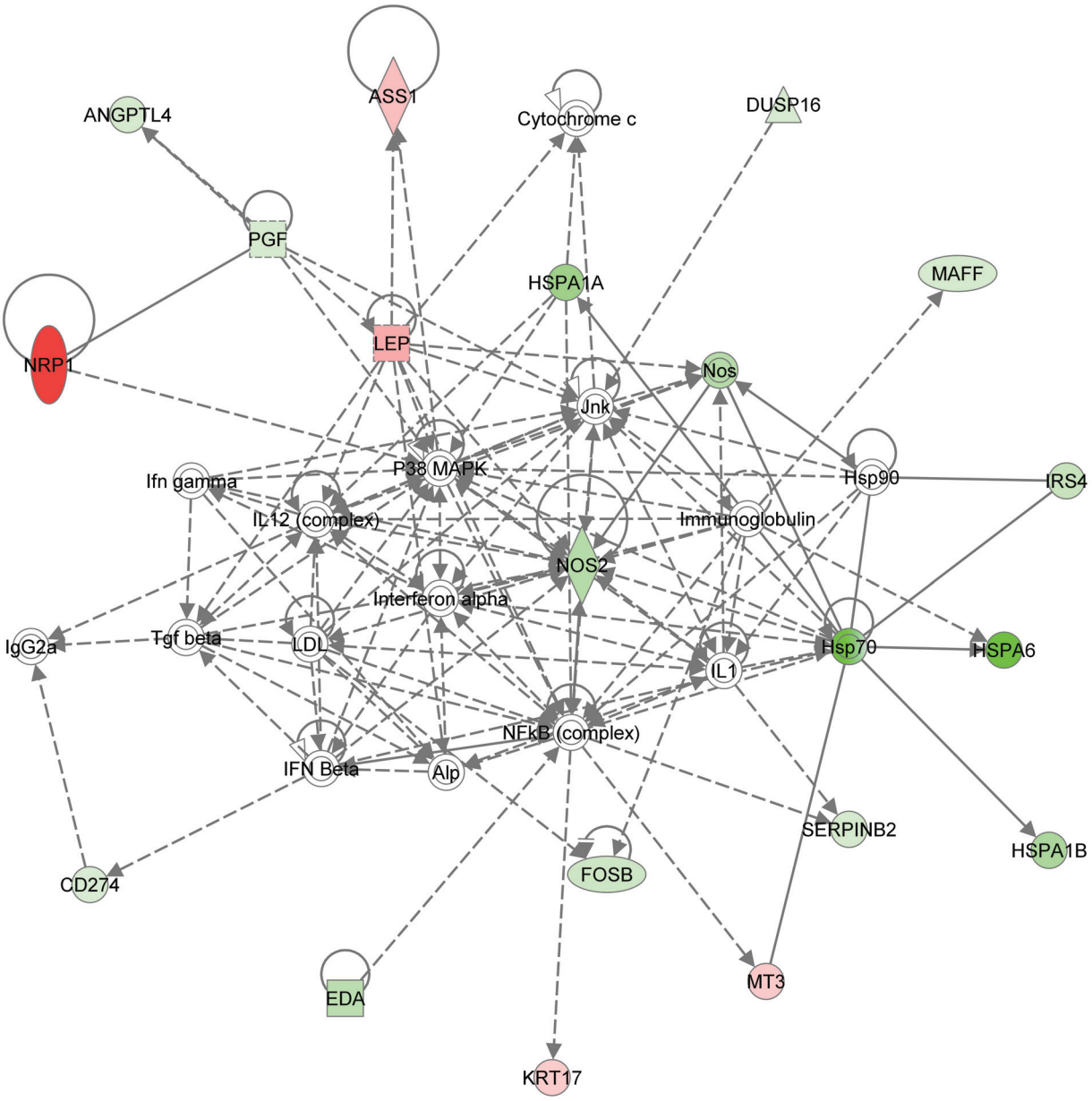
NM_0207151	PLEKHH1	pleckstrin homology domain containing, family H	-1.08	0.00005
NM_015597	GPSM1	G-protein signalling modulator 1	-1.08	0.01847
NM_001885	CRYAB	crystallin, alpha B	-1.08	0.00022
NM_000426	LAMA2	laminin, alpha 2 (merosin, congenital muscular dystrophy)	-1.08	0.00014
NM_181803	UBE2C	ubiquitin-conjugating enzyme E2C transcript variant 6	-1.08	0.00004
NM_030640	DUSP16	dual specificity phosphatase 16	-1.09	0.00161
NM_007280	OIP5	Opa interacting protein 5	-1.09	0.00055
NM_014875	KIF14	kinesin family member 14	-1.10	0.00061
NM_033379	CDC2	cell division cycle 2, transcript variant 2	-1.13	0.00018
NM_003504	CDC45L	CDC45 cell division cycle 45-like (S. cerevisiae)	-1.13	0.03153
NM_012323	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	-1.13	0.00012
NM_017853	TXNL4B	Homo sapiens thioredoxin-like 4B	-1.13	0.00059
NM_139314	ANGPTL4	angiopoietin-like 4 transcript variant 1	-1.13	0.00002
NM_014109	ATAD2	ATPase family, AAA domain containing 2	-1.14	0.00004
NM_004523	KIF11	kinesin family member 11	-1.14	0.00001
NM_002575	SERPINB2	serpin peptidase inhibitor, clade B member 2	-1.15	0.00004
NM_005375	MYB	v-myb myeloblastosis viral oncogene homolog	-1.15	0.00001
NM_002632	PGF	placental growth factor	-1.16	0.00604
NM_032417	SPANXD	Homo sapiens SPANX family, member D	-1.16	0.00568
NM_001211	BUB1B	BUB1 budding uninhibited by benzimidazoles 1	-1.18	0.00002
NM_022346	HCAP-G	condensation protein G	-1.21	0.00001
NM_178043	LARP2	La ribonucleoprotein domain family, member 2	-1.21	0.00527
NM_003671	CDC14B	CDC14 cell division cycle 14 homolog B	-1.21	0.00910
NM_020675	SPBC25	spindle pole body component 25 homolog	-1.23	0.03356
NM_057749	CCNE2	cyclin E2 (CCNE2), transcript variant 1	-1.26	0.00001
NM_145018	FLJ25416	hypothetical protein FLJ25416	-1.26	0.00001
NM_152782	SUNC1	Sad1 and UNC84 domain containing 1 transcript variant 2	-1.27	0.00019
NM_198537	FLJ44968	FLJ44968 protein	-1.27	0.00002

NM_006845	KIF2C	kinesin family member 2C	-1.28	0.00068
NM_018971	GPR27	G protein-coupled receptor 27	-1.28	0.00269
NM_001003786	LYK5	protein kinase LYK5 (LYK5), transcript variant 2	-1.28	0.00001
NM_006101	KNTC2	kinetochore associated 2	-1.28	0.00002
NM_006732	FOSB	FBJ murine osteosarcoma viral oncogene homolog B	-1.29	0.00003
NM_014264	PLK4	polo-like kinase 4 (Drosophila)	-1.30	0.00035
NM_001452	FOXF2	forkhead box F2 (FOXF2)	-1.31	0.00030
NM_006072	CCL26	chemokine (C-C motif) ligand 26	-1.31	0.00087
NM_020890	KIAA1524	KIAA1524	-1.34	0.00818
NM_019013	FAM64A	family with sequence similarity 64, member A	-1.34	0.00022
NM_0010341	RRM2	ribonucleotide reductase M2	-1.35	0.00086
NM_012310	KIF4A	kinesin family member 4A	-1.39	0.00008
NM_145665	SPANXE	SPANX family, member E	-1.39	0.00006
NM_1779371	GOLPH2	golgi phosphoprotein 2 (GOLPH2), transcript variant 2	-1.40	0.00419
NM_213590	RFP2	finger protein 2 (RFP2), transcript variant 3	-1.42	0.00009
NM_138340	ABHD3	abhydrolase domain containing 3	-1.43	0.00272
NM_016448	DTL	denticleless homolog (Drosophila)	-1.45	0.00000
NM_005573	LMNB1	lamin B1	-1.47	0.02333
NM_138419	FAM54A	family with sequence similarity 54, member A	-1.48	0.00000
NM_003604	IRS4	insulin receptor substrate 4	-1.50	0.01550
NM_003318	TTK	TTK protein kinase	-1.52	0.00003
NM_016084	RASD1	RAS, dexamethasone-induced 1	-1.53	0.00004
NM_006479	RAD51AP1	RAD51 associated protein 1	-1.56	0.00006
NM_198593	C1QTNF1	C1q and TNF related protein 1, transcript variant 2	-1.59	0.00006
NM_017669	FLJ20105	FLJ20105 protein (FLJ20105), transcript variant 1	-1.63	0.00009
NM_030919	C20orf129	chromosome 20 open reading frame 129	-1.66	0.00003
NM_172109	KCNQ2	potassium voltage-gated channel, KQT-like subfamily	-1.69	0.00238
NM_001005611	EDA	ectodysplasin A (EDA), transcript variant 4	-1.79	0.00099

NM_022661	SPANXC	SPANX family, member C	-1.84	0.00001
NM_018602	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	-1.84	0.00000
NM_172220	CSF3	Colony stimulating factor 3	-1.85	0.00000
NM_013453	SPANXA1	sperm protein associated with the nucleus, X-linked	-1.89	0.00001
NM_030926	ITM2C	integral membrane protein 2C, transcript variant 1	-1.94	0.00000
NM_153292	NOS2A	nitric oxide synthase 2A transcript variant 2	-1.95	0.00000
NM_032461.2	SPANXB1	SPANX family, member B1	-1.95	0.00000
NM_005252.2	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	-2.16	0.00002
NM_001002796	MCTP1	multiple C2 domains, transcript variant S	-2.23	0.00000
NM_005346.3	HSPA1B	heat shock 70kDa protein 1B	-2.27	0.00000
NM_139016.2	C20orf198	Chromosome 20 open reading frame 198	-2.29	0.00001
NM_018518.3	MCM10	MCM10 minichromosome maintenance deficient 10	-2.34	0.00001
NM_005345.4	HSPA1A	heat shock 70kDa protein 1A	-2.67	0.00000
NM_001012977	PAP1M	Polyadenylate-binding protein 1	-4.24	0.00000
NM_178539	FAM19A2	family with sequence similarity 19, member	-4.32	0.00000
NM_002155.3	HSPA6	heat shock 70kDa protein 6 (HSP70B')	-5.08	0.00000

Table S4: Primers used for real-time PCR experiments to detect the expression of TEL2, LEP, CSF3 and ANGPTL4 genes in human skeletal myoblasts infected with adenoviruses expressing GFP (Ad-GFP) and Ang-2 (Ad-Ang-2).

Gene			Accession #
TELO2	Forward	5'-GACCTGGACAGCGATGATGA-3'	NM_016111
	Reverse	5'- CCATCTTCATCAGCCGGAAG-3'	
LEP	Forward	5'- TGTGGCTTTGGCCCTATCTT-3'	NM_000230
	Reverse	5'- AGCCCAGGAATGAAGTCCAA-3'	
CSF3	Forward	5′-CGCTCCAGGAGAAGCTGT-3′	NM_000759
	Reverse	5′-CCAGAGAGTGTCCGAGCAG-3′	
ANGPTL4	Forward	5'-CCACTTGGGACCAGGATCAC-3'	NM_139314
	Reverse	5'- CGGAAGTACTGGCCGTTGAG-3'	



Supplementary Figure 1: The second top network of regulated genes in human myoblasts infected with Ad-ANGPT2 viruses vs. cells infected with Ad-GFP viruses (control condition). This figure was created with the Ingenuity Pathway Analysis system. Symbols are identical to those shown in supplementary figure 1. Top functions associated with this network include tissue morphology, lipid metabolism and small molecule biochemistry.

PREFACE TO CHAPTER 4

In chapter 3, I addressed the functional role of Ang-2 in regulating the myogenesis program of skeletal myoblasts and reported that Ang-2 is produced by skeletal myoblasts and that exposure of these cells to exogenous Ang-2 stimulates myoblast survival, inhibits apoptosis and strongly enhances skeletal myoblast differentiation into myotubes. I didn't address in chapter 3 whether these actions of Ang-2 on skeletal myoblasts are mediated through Tie2 receptors and whether Ang-1 produces similar effects on skeletal myogenesis program to those elicited by Ang-2.

Very little is known about the expression of Tie-2 receptors in skeletal muscle cells. Dallabrida *et al.* (13) failed to detect these receptors in primary skeletal myoblasts from humans and concluded that the adhesion of these cells to Ang-1 protein is mediated through a selective interaction between integrins and Ang-1. More recently, Abou-Khalil *et al.* have reported that Tie2 is present in a subpopulation of skeletal muscle progenitors known as Regenerating Cells and that these receptors help maintain the population of these cells (2). It is unclear whether Tie2 receptors play any role in skeletal muscle myogenesis and in the repair of skeletal muscles from injury.

In chapter 4, I investigated in details the functional significance of Ang-1 and Tie2 receptors in the activation and differentiation of skeletal muscle progenitors in *in-vitro* settings. I also assessed the effectiveness of Ang-1 in promoting the *in-vivo* regenerative capacity of injured skeletal muscle in mice. My results reveal for the

first time an important role for the Ang-1/Tie2 axis in promoting differentiation of skeletal muscle progenitors and for the enhancement of *in-vivo* regenerative capacity of injured skeletal muscle.

CHAPTER 4

ANGIOPOIETIN-1 PROMOTES MUSCLE REGENERATION AND MYOBLAST DIFFERENTIATION THROUGH COMPLEMENTARY PATHWAYS INVOLVING TIE2

ANGIOPOIETIN-1 PROMOTES MUSCLE REGENERATION AND MYOBLAST DIFFERENTIATION THROUGH COMPLEMENTARY PATHWAYS INVOLVING TIE2

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4.1 ABSTRACT

The inherent regulation of both capillary density and muscle precursor cells is critical to muscle repair or re-growth from injury, but it is unknown whether endothelial and muscle cells respond to similar stimuli during recovery. Recent studies have identified traditional endothelial cell receptors in skeletal muscle cells, but their roles in muscle plasticity remain controversial. We examined the role of the receptor tyrosine kinase Tie2 and its ligand angiopoietin-1 (Ang-1) in regenerating skeletal muscle *in vivo* and during the muscle cell maturation process *in vitro*. QRT-PCR and immunofluorescence of primary satellite cells isolated from mTmG-Tie2Cre mice *in vitro* confirmed that Tie2 is expressed in skeletal muscle cells, and this expression increases with differentiation. Human and mouse muscle cells also expressed Ang-1 and treatment of myoblasts with recombinant Ang-1 enhanced cell proliferation, survival, and the subsequent transcriptional differentiation program through induction of the myogenic regulatory factors MyoD and myogenin, resulting in increases in myosin heavy chain (MHC) protein abundance. Inhibiting Tie2 with adenoviruses expressing soluble Tie2 (Ex-TEK) attenuated Ang-1 induced myoblast proliferation. Transcriptional profiling of Ad-Ang-1 infected human muscle cells *in vitro* revealed increases in cell cycle regulator (TEL2), vascular growth factor receptor (neuropilin-1/NRP-1), and chemokine (CXCL2) genes as well as decreases in genes involved in inhibiting angiogenesis (SERPINB2 and SERPINC1) and protein folding (Heat Shock 70kDa). *In vivo*, adenoviral overexpression of Ang-1 enhanced tibialis anterior muscle regeneration by activating and subsequently driving differentiation of the muscle satellite cell population to rapidly restore muscle function, and Tie2 protein

co-localized to centralized nuclei of regenerating myofibres. Myoblasts stably expressing shRNA for Tie2 ($Tie2^{low}$) demonstrated deficits in the expression of p21, MyoD, and myogenin during the later stages of maturation and exogenous Ang-1 rescued these deficits. The catalytic activity of Tie2 in differentiating myoblasts appears to be a time-dependent requirement for full maturation, however, as transient transfection enabling expression of full length human Tie2 after 48-hours of differentiation rescued a portion of the defective phenotype in $Tie2^{low}$ myoblasts, while similar transfection of a catalytically dead truncated mutant human Tie2 did not. Our results indicate that Ang-1 signaling enhances skeletal muscle regeneration through multiple receptor pathways, including Tie2, resulting in stimulation of the muscle precursor cell activation and differentiation program.

4.2 INTRODUCTION

Skeletal muscle has an exceptional capacity to recover after injury. This plasticity involves the coordination of processes involved in vascular growth (angiogenesis) and maintenance, extracellular matrix deposition, and nerve and muscle fibre regeneration (10, 18, 50). Current pharmacological therapies targeting patients with injured limb muscles are inefficient, as they are largely directed toward individual cell types or specific signaling pathways. The development of therapeutic strategies to enhance recovery in multiple cell types would likely improve clinical outcomes. Despite differences in the transcriptional responses of the various cell types in skeletal muscle tissue (4), increasing evidence demonstrates that their responses to injury are critically linked through regulatory factors traditionally thought to target blood vessels exclusively. The vasculature plays an important role in the regulation of metabolism and protein composition in skeletal muscle, and skeletal muscle cells secrete angiogenic factors in response to changes in metabolic demand or stress (4, 14, 19, 40). However, the mechanisms of cross-talk between skeletal myocytes and endothelial cells during periods of injury and stress remain poorly defined.

The capacity for muscle tissue repair is conferred by the satellite cells located between the basal lamina and the sarcolemma of mature myofibres. Satellite cells are quiescent myoblasts that play critical roles in muscle hypertrophy, recovery from disuse, and regeneration after injury (34, 38). Following muscle injury, quiescent satellite cells re-enter the cell cycle, proliferate to repopulate the satellite cell pool, and give rise to a large number of daughter myogenic precursor cells before they fuse

to existing myotubes or contribute to the regeneration of necrotic myofibres (43). Intrinsic control of satellite cell activity is regulated by coordinated signaling through helix-loop-helix myogenic regulatory factors (MRFs), including MyoD, myf-5, myogenin, MRF4, and cell cycle regulatory factors such as cyclin D1 and p21 (42). MyoD and myogenin are specifically associated with skeletal muscle precursor cells and regulate proliferation, differentiation, and cell cycle withdrawal upon commitment to differentiation (8). Satellite cells reside in close proximity to myofibre capillaries and receive support from endothelial cells in the form of various growth factors during activation, including basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) during activation. In return, satellite cells produce endothelial growth factors to stimulate angiogenesis to provide the new tissue with oxygen and nutrients (12, 21). It remains unclear the extent to which traditional "vascular" growth factors also play a role in the determination of skeletal myocyte survival, recovery from insult, and regulation of cellular metabolic pathways.

Among the early genes expressed in response to skeletal muscle injury are several angiogenic growth factors, including vascular endothelial growth factor (VEGF) and the angiopoietins (Ang-1, Ang-2), and their cognate receptors (47). Like VEGF, the effects of the angiopoietins are not specific for vascular endothelial cells, as their receptors (Tie1, Tie2) are expressed in hematopoietic cells and have recently been shown expressed in skeletal muscle cells (2, 11). Dallabrida *et al.* demonstrated that Angiopoietin-1 (Ang-1) exerts protective effects on both cardiac and skeletal

muscle cells through activation of integrins and downstream focal adhesion complex-mediated survival pathways (13). Expression of Tie receptors on myocytes was not observed in that study, but the effects of Ang-1 were instead linked to integrin signaling. Integrin heterocomplexes, particularly those involving β1 integrin, are well known to play critical roles in skeletal muscle function and maturation (27, 28, 32, 36, 52). Interestingly, Ang-1 signaling in endothelial cells has recently been shown to be at least partly dependent on formation of a complex between Tie2 and α5β1 integrin (9). However, a role for Ang-1-Tie2 signaling in myocytes has not been well studied. Abou-Khalil *et al.* recently demonstrated a pivotal role for Tie2 in the Ang-1-induced maintenance of quiescence in a sub-population of precursor reserve cells (2). Although an increase in Tie2 expression during myoblast differentiation was shown in that report, a biological role for Ang-1/Tie2 signaling in the myotube maturation process was not described (2). Angiopoietin/Tie receptor signaling in the endothelium is known to be context-dependent (15), thus it is possible that this ligand-receptor system could have distinct effects on specific populations of muscle precursor cells (i.e., quiescent, proliferating, or differentiating cells)(2, 7, 14, 30, 37), and examining effects at other developmental stages may be important to elucidate the role of Ang-1/Tie2 signaling in skeletal muscle.

In this study, we examined the role of Ang-1/Tie2 in muscle precursor cell activation and differentiation as well as in skeletal muscle regeneration. We show that skeletal muscle Tie2 protein is expressed in regenerating muscle fibres *in vivo* and is required for sustained satellite cell activation, differentiation, and myotube maturation *in vitro*. We also demonstrate that exogenous Ang-1 induces the

proliferation and differentiation of muscle precursor cells *in vitro* and enhances both capillary density and muscle cell regeneration following cardiotoxin-induced injury *in vivo*. The myogenic effects of Ang-1 appear to be due to signaling through multiple receptor complexes in myocytes, as exogenous Ang-1 rescues the myogenic differentiation program in Tie2-deficient satellite cells. Together, these findings point to a novel and previously unrecognized role for Ang-1/Tie2 signaling in skeletal muscle regeneration following injury.

4.3 METHODS

4.3.1 ANIMAL STUDIES

Animal experiments were approved by both the McGill University Animal Ethics Committee in accordance with standards established by the Canadian Council of Animal Care and the Duke University Institutional Animal Care and Use Committee. Adult male C57BL/6J mice (6- 8 weeks old) were obtained from Jackson Laboratories. To investigate the role of angiopoietin/Tie2 signaling in skeletal muscle regeneration, we used the cardiotoxin injury model, essentially as described previously (54). Mice were anesthetized with ketamine (130mg/kg) and xylazine (20 mg/kg) by intraperitoneal injection. Lower limbs were then shaved and cardiotoxin (10 μM in 50 μl PBS, Sigma) was injected into one tibialis anterior (TA) muscle to induce skeletal muscle injury in vivo, as described previously (24). The contra-lateral TA muscle was injected with 50 μl of phosphate buffered saline (PBS) and served as an uninjured control. Mice were euthanized by anesthetic overdose on days 1, 3, 7 and 14 after cardiotoxin injection. Additional animals were utilized to assess effects of Ang-1 over-expression on recovery of muscle contractility following necrotic injury. These mice underwent cardiotoxin injection as above and were then allowed to recover with free access to water and food for four days. Animals were then anesthetized and the injured TA muscle was injected with 30 μl of recombinant adenovirus (1.5×10^9 viral particles/muscle) encoding human Ang-1 (Ad-Ang-1) or GFP (Ad-GFP) as a control. The contralateral muscle was left un-injured and served as an additional control. Six or 10 days later (10 or 14 days post injury, respectively), mice were anesthetized, immobilized in the supine position, and contractility was

measured *in situ* in the TA muscles bilaterally. Muscles were then harvested for histological or immunohistochemical or immunofluorescent analysis.

Transgenic B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (mTmG) mice were provided by Dr. Steven Crowley at Duke University Medical Center and have been described previously (35). These mice possess a double-fluorescent Cre reporter consisting of membrane-targeted tandem dimer Tomato (mT) expressed at baseline and membrane-targeted green fluorescent protein (mG) expressed following Cre-mediated excision. Cre-mediated recombination to homozygosity was driven by crossing male mTmG mice with homozygous female Tie2-Cre transgenic mice (B6.Cg-Tg(Tek-cre)1Ywa/J, Jackson Labs). Upon activation of the Tie2 promoter, Cre recombinase excises the mTomato gene, inducing mGFP expression and allowing visualization of Tie2 promoter activity at specific developmental time points. Myoblasts were isolated from these mice, as described below, and used to demonstrate activation of muscle Tie2 expression during myotubes differentiation.

4.3.2 TIBIALIS MUSCLE CONTRACTILITY

Both legs were secured to a surgical platform at the knee and ankle with two 27.5 gauge needles. The distal tendon of the tibialis anterior (TA) muscle was isolated and tied with fine silk suture to the lever arm of a force transducer/length servomotor system (model 305B dual mode; Cambridge Technology, Watertown, MA) mounted on a mobile micrometer stage to allow fine adjustment of muscle length. The exposed portion of the TA was kept moist with a 37°C isotonic saline drip, and the muscle was then stimulated directly via an electrode placed on the belly of the

muscle. Supramaximal stimuli (2 ms pulse duration) were delivered using a computer-controlled electrical stimulator (model S44; Grass Instruments, Quincy, MA). Muscle force and length signals were displayed on a storage oscilloscope (Tektronix, Beaverton, OR) and simultaneously acquired to a computer (Labdat/Anadat software; RHT InfoData, Montreal, Quebec) via an analog to digital converter at a sampling rate of 1000 Hz. After adjusting the TA to optimal muscle length (L_o, the length at which maximal twitch force is achieved), two twitch stimulations were recorded, and the mean value was taken as the maximal isometric twitch force. The force-frequency relationship was then determined by sequential supramaximal stimulation for 300 ms at 10, 30, 50, 100, and 120 Hz, with 2 min between each stimulation train. After completion of contractility studies, the muscle length was measured with a microcaliper accurate to 0.1 mm and the muscle was weighed. Muscle force was normalized to tissue cross-sectional area, which was determined by assuming a muscle density of 1.056 g/cm^3. Specific force (force/cross-sectional area) was expressed in Newtons/cm^2.

4.3.3 MUSCLE MORPHOLOGY

Five-µm-thick transverse sections were cut from the middle region of each muscle. Sections were stained with hematoxylin and eosin and were used to detect three indices of muscle regeneration: fibre cross-sectional area (CSA); non-regenerated (injured) and regenerated fraction; and the number of nuclei per regenerated fibre. These parameters were calculated by analysis of up to 250 fibres per section. Images of muscle cross sections were captured with a CCD digital camera (Retiga 2000R, Q Imaging, Surrey, British Columbia) mounted on an inverted microscope (model

IX70, Olympus, Tokyo, Japan) and analyzed using computerized image analysis software (Image Pro Plus, MediaCybernetics, Bethesda MD).

4.3.4 IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemistry was performed with the UltraVision LP Value Detection System (AP polymer and Fast Red Chromatogen, Thermo Scientific, Fremont, CA). Rehydrated muscle sections underwent antigen retrieval by exposure to sodium citrate buffer at 95-100ºC for 20 min. After cooling, sections were incubated in 0.1% Triton X-100 for 15 min, rinsed, blocked with Ultra V Block, and then incubated overnight at 4ºC with primary antibodies against Ang-1 and Ang-2 (R&D Systems, Minneapolis, MN), embryonic myosin heavy chain (Developmental Studies Hybridoma Bank [DSHB], University of Iowa) and von Willebrand Factor (vWF, Chemicon International, Temecula, CA). After washing, the sections were then incubated with Primary Antibody Enhancer (20 min) and Value AP Polymer anti-mouse/rabbit secondary antibodies (20 min). Positive staining was visualized using Fast Red and counterstained with hematoxylin.

For immunofluorescence, eight-µm-thick transverse sections were cut from TA frozen in optimum cutting temperature (OCT) medium. Sections were allowed to come to room temperature and fixed/permeabilized with ice-cold acetone for 10 min at 4°C. Sections were allowed to air dry for 5 min at room temperature (RT) and rehydrated in PBS before blocking in 5% normal goat serum (Sigma) in PBS for 45 min at RT. Slides were then incubated overnight at 4°C in a primary antibody solution of rat anti-CD31 (Abd Serotec MCA-1364), mouse anti Tie2 (Ab33), and mouse anti-dystrophin (MANDYS1 3B7, DSHB) in blocking solution. Slides were

then washed with PBS and incubated with species- and isotype-specific secondary antibodies (Alexa Fluor 488 goat anti-rat IgG, Alexa Fluor 568 goat anti-mouse IgG1, and Alexa Fluor 633 goat anti-mouse IgG2a, all from Invitrogen) in blocking solution. Sections were then washed with PBS and mounted using Vectashield HardSet Mouting Medium with DAPI (Vector Labs H-1500). Images were captured on a Zeiss Axio Observer 510 Inverted Laser Scanning Microscope (LSM) using the 405 nm Diode, Argon/2 (458, 477, 488, 514nm), 561nm Diode, and HeNe 633nm lasers using the Zeiss LSM 510 software (v. 4.2). Images were analyzed offline using ImageJ image analysis software (v. 1.44o, NIH).

4.3.5 PRIMARY MYOBLAST ISOLATION AND CELL CULTURE

Primary human muscle precursor cells (human myoblasts) immortalized by expression of human papillomavirus E6E7 were generously provided by Dr. E. Shoubridge (McGill University, Montréal, Quebec) and cultured in SkBM medium (SkBM Bullet Kit, Cambrex, East Rutherford, NJ) supplemented with 15% inactivated fetal bovine serum (FBS) (33). To induce differentiation into myotubes, 90% confluent human myoblasts were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 2% inactivated horse serum (HS) for 6 days. Differentiation was evaluated by immunoblotting, by assessment of cellular morphology, and by expression of myogenin, myosin heavy chain, and creatine kinase. Primary murine muscle precursor cells (mouse myoblasts) derived from diaphragm and hindlimb muscles were prepared as described by Rosenblatt *et al*. (41). Briefly, muscle strips were extracted from 6-week-old C57BL/6 mice, digested with collagenase (0.2% at 37°C for 60 min), and then triturated to break

muscle tissues into single fibres. Individual fibres were washed in DMEM and PBS, transferred into matrigel-coated (1 mg/ml in DMEM) 6-well plates and maintained for 4 days in DMEM supplemented with 10% HS, 0.5% chick embryo extract (MP Biomedicals, Aurora, OH) and containing 1% penicillin/streptomycin and 0.2% amphotericin B, which allowed myoblasts to adhere to the substratum. Myoblasts were then grown in growth medium (DMEM supplemented with 20% FBS, 10% HS, 1% chick embryo extract) for 6 days. Identical growth medium was used to maintain immortalized murine C2C12 cells. Human umbilical vein endothelial cells (HUVECs) and HEK 293 cells were used as positive and negative controls, respectively, for angiopoietin and Tie2 expression and Tie2 phosphorylation. HUVECs were maintained as described previously (20). To analyze Tie2 phosphorylation, cells were serum-starved overnight and then treated for 15 min with either PBS alone or recombinant human Ang-1 (300 ng/ml) in PBS. HEK 293 cells (Clontech) were maintained in DMEM supplemented with 10% FBS and antibiotics.

4.3.6 GENERATION OF STABLE TIE2^{low} MYOBLASTS AND TRANSIENT TRANSFECTIONS

Primary myoblasts from C57BL/6 mice were used to generate scrambled control (Tie2^{Scr}) and Tie2 knockdown (Tie2^{low}) stable myoblast lines using retroviral infection as previously described (5), with the following modifications. shRNAs targeting mouse Tie2 (AACCAGCTGTGCAGTTTAACT and AAGGGACTTTGAAGCCTTAAT) and a control scrambled shRNA (GGAATCTCATTCGATGCATAC) were obtained from SABiosciences. Tie2-targeting constructs were validated for efficient Tie2 silencing by transient co-

transfection with murine Tie2 into HEK 293 cells and then subcloned into pSuper-Retro (OligoEngine). Recombinant ecotrophic retroviruses were generated by transfection into Phoenix-Eco packaging cells (provided by Dr. Gary Nolan, Stanford University (26)), as described previously (16), and used to infect primary rat BL6 myoblasts. Myoblasts stably expressing retroviral constructs were selected with puromycin (1 μg/ml). Generation of Tie2 rescue myoblasts was performed by the construction of retroviral hTie-2 virus using Phoenix cells, as described above. hTie2 rescue retrovirus was then used to infect retroviral stable Tie-2^{low} myoblasts. After 48-hours, puromycin supplemented GM was supplemented with 600μg/ml neomycin and secondary selection was induced. Verification of knockdown and rescue was performed using QRT-PCR (TaqMan, Applied Biosystems) and immunoprecipitation-immunoblotting. Transient transfections of differentiating $Tie2^{low}$ myoblasts were performed using Lipofectamine 2000 (Invitrogen). Briefly, $Tie2^{low}$ myoblasts were plated at equal density, allowed to reach confluence, and switched to differentiation medium as described above. After 24-hours of differentiation, myoblasts were transfected for 2-hours with either GFP, full-length human Tie2 (LNCX-hTie2), or a human Tie2 mutant truncated after the transmembrane (TM) domain and lacking the entire cytosolic domain (designated Tie2TM). The mutant was created by PCR and incorporated a stop codon immediately after the last residue of the transmembrane domain (isoleucine 782). PCR was also used to generate new Not I and BsiW I restriction sites (underlined): forward primer, 5'-TAGCGGCCGCGTATGGACTCTTTAGC-3'; reverse primer, 5'-GGACGTACGATCTAGGTACCTACGCT-3'. The Tie2TM construct was cloned

into the pQCXIH plasmid (Clontech). Cell transfections were repeated at 48-hours of differentiation.

4.3.7 NON-RADIOACTIVE NORTHERN BLOTTING

Total RNA was extracted from human and murine skeletal myoblasts and HUVECs (all grown in growth medium) using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Oakville, Ontario). Five to 7.5 µg of total RNA were loaded on a 1.2% agarose-formaldehyde gel. After electrophoresis, RNA was transferred to nylon membranes (Roche Diagnostics Canada, Laval, Quebec, Canada), and digoxigenin-labeled (Roche) human Ang-1 and Ang-2 cDNA probes were hybridized overnight at 42°C in an ULTRAhyb buffer (Ambion, Inc., Austin, Tex). An 18S rRNA probe was used as a loading control. Signal was detected using a digoxigenin luminescent detection kit (CDP-*Star*, Roche) according to the manufacturer's instructions.

4.3.8 RT-PCR AND QRT-PCR

Total RNA was extracted from TA muscles and from cultured cells using either TRIzol reagent (Invitrogen) or the GenElute Mammalian Total RNA Miniprep Kit. RNA (2- or 5µg) was reverse-transcribed using Superscript II Reverse Transcriptase and random primers (Invitrogen). Conventional RT-PCR was used to amplify the entire coding sequence of Tie-2 mRNA transcripts in human skeletal myoblasts. PCR was performed using oligonucleotide primers designed to amplify the entire coding sequence of Tie-2 mRNA (forward 5'-ATGGACTCTTTAGCCAGCTT-3' and reverse 5'- CTAGGCCGCTTCTTCAGCA-3', 3374 base pair amplicon size). The experimental conditions for PCRs were as follows: 94º for 2 min (initial denaturation)

followed by 35 cycles of 94 º C (20 s), 55 º C (30s) and 68º C (1 min), and a final extension reaction for 10 min. To verify the accuracy of the amplified sequence, PCR products were sequenced at the McGill University DNA Sequencing Facility. In addition, PCR generated products were sequenced (Sheldon Centre, McGill University), subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. Real-time PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Expression of myogenic transcription factors MyoD and myogenin, muscle-specific myosin heavy chain, and Tie-2 were detected using real-time PCR as follows. Primers designed to amplify human MyoD (Forward 5'- TCTCTGCTCCTTTGCCACAA-3' and Reverse 5'-AGTGCTCTTCGGGTTTCAGG-3'), myogenin (Forward 5'-GAAGGTGAATGAGGCCTTCG -3' and Reverse 5'-AGGCGCTCGATGTACTGGAT-3') and Myosin Heavy Chain (Forward 5'-AGCTGGACGAAAGGCTCAAG-3' and Reverse 5'-CATAGTCGCTGCGCTGTTTC -3'), were used. For human MyoD, myogenin, mMyosin heavy chain and 18S mRNA transcripts, standard curves that related the cycle threshold (C_T) values of these genes to the copy numbers were established. Copy numbers of these genes were then calculated using these curves and were normalized per copies of 18S. In addition, relative quantification of mouse and human Tie-2, MyoD, Paired Box gene (Pax7), myogenin, p21, Pgc1-α, creatine kinase (CK), Ang-1, Ang-2, Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM1), Tie-1, β-Actin and GAPDH mRNA levels were determined using the comparative threshold cycle (ΔΔCT) method using either TaqMan Gene Expression

Assays (Applied Biosystems) specific to these genes or 25µl of SYBR Green PCR Master Mix (Qiagen Inc., Valencia, CA) and 3.5µl of each 10µM primer (listed in Table 1). A melt analysis for each SYBR Green PCR experiment was used to assess primer-dimer formation or contamination.

4.3.9 IMMUNOBLOTTING

Immunoblotting was performed using standard procedures. Cultured cells were lysed with either Triton or RIPA lysis buffer, and lysates were blotted with the following antibodies: Ang-1 (R&D Systems Inc.), phospho-FAK1 (Tyr^{397}), total FAK1, phospho-Paxillin (Tyr^{31}), phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and total ERK1/2, phospho-p38 (Thr^{180}/Tyr^{182}), phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), total SAPK/JNK, phospho-AKT (Thr^{308}), total AKT, phospho-mTOR (Ser^{2481}), total mTOR, phospho-p70SK1 (Thr^{389}), total p70SK1, phospho-4E-BP1 ($Thre^{37/46}$), total 4-E-BP1, and phospho-c-Jun (Ser^{63} and Ser^{73}) (all from Cell Signaling Inc.), MyoD (Santa Cruz Biotechnology), myogenin (A4.74), myosin heavy chain (MF20, both from the Developmental Studies Hybridoma Bank, University of Iowa), total Tie2 (C-20, Santa Cruz Biotechnology, or Ab33), phospho-Tie2 ($pTyr^{1102}/pTyr^{1108}$, Ab1, Calbiochem, or pTy^{992}, Cell Signaling), and anti-phosphotyrosine (4G10, Upstate Biotechnology). Loading and transfer of equal amounts of protein was confirmed by ponceau staining and probing with anti-α-tubulin (Sigma-Aldrich) or -GAPDH (Novus Biologicals).

4.3.10 TIE2 PHOSPHORYLATION IN MYOBLASTS

Human myoblasts (1×10^5 cells) were seeded into wells of 6-well plates for 24h in SkBM culture medium containing 15% FBS. The medium was then replaced with

SkBM culture medium containing 5% FBS for 6h, and the cells were treated with PBS or recombinant human Ang-1 (600 ng/ml) for 15 or 60 min. Cells were lysed and Tie2 phosphorylation was detected by immunoblotting with anti-phospho-Tie2 or by immunoprecipitation with monoclonal anti-Tie2 (Ab33) followed by blotting with anti-phosphotyrosine (4G10). Equal protein loading was demonstrated by probing for total Tie2 in both cases.

4.3.11 MYOBLAST PROLIFERATION

The effects of Ang-1 and Ang-2 on myoblast cell proliferation were measured using two different assays: cell counts and bromodeoxyuridine (BrdU) incorporation. Human myoblasts (1×10^5 cells) were seeded into wells of 6-well plates for 24h in SkBM culture medium containing 15% FBS. Culture medium was then replaced with SkBM culture medium containing 5% FBS and either PBS or recombinant human Ang-1 or Ang-2 at the indicated concentrations. Culture medium containing PBS, Ang-1, or Ang-2 was replenished every 24h. After 96h of exposure, cells were then trypsinized and viable cells were counted on a hemotocytometer following trypan blue exclusion. We also evaluated the effects of adenovirus-mediated over-expression of Ang-1 or Ang-2 on myoblast proliferation. Generation of adenoviruses expressing green fluorescent protein (Ad-GFP)(control condition), human Ang-1 (Ad-Ang-1), and human Ang-2 (Ad-Ang-2) has been described previously (46). Human skeletal myoblasts (1.5×10^5 cells) were seeded in 24-well plates and infected for 5h with Ad-GFP, Ad-Ang-1, or Ad-Ang-2 in basal medium. These conditions resulted in uniform expression of transgenes in ~90% of cells as assessed by GFP fluorescence. Viruses were then removed and cells were allowed to recover in

complete medium for 48h. Cell proliferation was measured as described above by seeding an equal number of GFP-, Ang-1- and Ang-2-expressing myoblasts (1×10^5 cells) into 6-well plates in SkBM culture medium containing 5% FBS. Viable cell number was counted 72h later. For BrdU incorporation assays, a Cell Proliferation ELISA, BrdU colorimetric kit was used (Roche Applied Science, Laval, Quebec). Myoblasts infected with Ad-GFP, Ad-Ang-1 and Ad-Ang-2 viruses were plated into 96-well plates at a density of 5×10^3 cells/well in 100 μl of SkBM culture medium containing 5% FBS. After 72h, cells were pulse-labeled for 4h with 10 μM BrdU, fixed and then detected according to the manufacturer's instructions. Absorbance (370nm) was measured 10min after the addition of substrate. To inhibit the effects of Ang-1 on myoblast proliferation, we used an adenoviral vector encoding soluble Tie2 (Ad-Ex Tek)(31). Myoblasts were infected with Ad-GFP (control virus) or Ad-Ex Tek as described above. After 48h of recovery, cell proliferation was measured as described above by seeding equal number of GFP-, and Ex Tek-expressing myoblasts (1×10^5 cells) into 6-well culture plates in SkBM culture medium containing 5% FBS in the presence of PBS or 600 ng/ml of recombinant human Ang-1. After 72h of culture, viable cells were counted as described above.

4.3.12 MYOBLAST SURVIVAL AND APOPTOSIS

To evaluate the influence of Ang-1 on skeletal muscle precursor cell survival, human skeletal myoblasts (2.5×10^4 cells) were seeded into 96-well plates and maintained for 12h in SkBM culture medium containing 15%FBS. Culture medium was then replaced with serum-free SkBM containing either PBS or recombinant human Ang-1 (600 ng/ml). Cytotoxicity and caspase3/7 activity were measured 36h later using

CytoTox-Fluor Cytotoxicity Assay and Caspase-Glo 3/7 Assay, respectively, according to the manfacturer's instructions (Promega Inc. Madison, WI).

4.3.13 MYOBLAST MIGRATION

Effects of Ang-1 and Ang-2 on skeletal myoblast migration were assessed using an *in vitro* scratch assay. Human myoblasts (1×10^5 cells) were seeded into 6-well culture plates and maintained for 24h in SkBM culture medium containing 15% FBS. Cells were then carefully wounded using a 200-□l pipette tip, as described previously (1). Cellular debris was removed by washing with PBS. After wounding, culture medium was replaced with SkBM containing 5% FBS and either PBS (control) or recombinant human Ang-1 or Ang-2 (600 ng/ml). Wounds were photographed immediately after wounding (time = 0) and 8, 12, and 18h later using an Olympus inverted microscope (Model IX70) equipped with phase-contrast objectives. Migration was evaluated by measuring the reduction in the width of the wound after migration of the cells into the cell-free zone (1). For each condition, triplicate wells were analyzed, and each experiment was performed in triplicate. In addition, we evaluated the influence of adenovirus-mediated overexpression of Ang-1 or Ang-2 on myoblast migration. Myoblasts were infected with Ad-GFP, Ad-Ang-1, or Ad-Ang-2 as described above. After 48h of recovery, cells were seeded for 24h in 6-well plates and were then wounded with a pipette tip, and migration was assessed 12h later as described above.

4.3.14 MYOGENIN PROMOTER ACTIVITY

To further evaluate the influence of Ang-1 on muscle differentiation, we evaluated the influence of Ang-1 on murine myogenin promoter activity. A 660 bp fragment of

the mouse myogenin promoter was PCR cloned from mouse genomic DNA and cloned into a firefly luciferase reporter plasmid (Myogenin-pGL3-Luc). Generation of MyoD-pcDNA3.1 expression vector was described previously (25). The human Ang-1 cDNA was cloned in-frame with and upstream of GFP in the pEGFP-N1 vector (Clontech Inc.) to generate pAng-1-N1, while empty pEGFP-N1 vector was used as control. Human skeletal muscle myoblasts or C2C12 myoblasts were transiently transfected with empty PGL3-Luc or Myogenin-pGL3-Luc vectors in the presence or absence of MyoD-pcDNA3.1, pAng-1-N1, or pEGFP-N1 vectors using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen Inc.). Cells were lysed 48h later, and firefly luciferase activity was measured using the Dual Luciferase Assay Kit (Promega Inc.) and normalized to relative firefly luciferase activity.

4.3.15 ANG-1-INDUCED GENE EXPRESSION

To investigate the effects of Ang-1 on myocyte gene expression, we infected human skeletal myoblasts with Ad-Ang-1 or Ad-GFP and compared mRNA expression profiles from these cells using microarrays. Cells were infected with Ad-GFP or Ad-Ang-1 as described above, and after 48h of recovery in complete culture medium, the culture medium was changed to SkBM culture medium containing 5% FBS. Total RNA was harvested 12h later 100 ng was amplified using the Illumina RNA Amplification kit (Illumina Inc., San Diego CA) and labelled by incorporation of biotin-16-UTP. Samples were hybridized to Sentrix Genome-Wide Expression BeadArrays (Illumina Inc.). These arrays use beads containing 50-mer gene-specific probes (a total of 46,000 probes per array). Arrays were scanned with an Illumina

BeadArray Reader (Montreal Genome Centre, Montreal, Canada) and data processing and normalization were performed using Illumina Bead-Studio software. Signal values were normalized by global mean and log-transformed using GeneSifter software (VizX Labs, Seattle, WA, USA). Pairwise comparisons and Student's t-test were subsequently performed using the FlexArray 1.1.3 software package (Montreal Genome Centre, Montreal, Canada), and a difference of at least two-fold with a P-value of less than 0.05 was considered statistically significant. To investigate gene networks regulated by Ang-1, we analyzed the microarray results using Ingenuity Pathway Analysis, a web-based bioinformatics tool (IPA, http://www.ingenuity.com). Gene name identifiers or Ingenuity probe set IDs were uploaded into IPA and queried against all other genes stored in the IPA knowledge database. Each Ingenuity probe set ID was mapped to its corresponding gene identifier in the IPA knowledge database. Probe sets representing genes having direct interactions with genes in the IPA knowledge database are called ‘‘focus’’ genes, which are then used as a starting point for generating functional networks. Each generated network is assigned a score according to the number of differentially regulated focus genes in our array dataset. These scores are derived from negative logarithm of the P indicative of the likelihood that focus genes found together in a network due to random chance. Scores of 14 or higher have 99.9% confidence level of significance. In reporting our findings we list networks with a substantially higher confidence limit and thus represent strong evidence for a given biological pathway being regulated by Ang-1 in human skeletal myoblasts. It should be noted however that while the database extends the interpretation beyond mRNA transcript levels (as network genes don’t have to be

differentially expressed at the mRNA level) the database is finite and reflects current knowledge.

4.4 RESULTS

4.4.1 SKELETAL MUSCLE EXPRESS ANG-1-RESPONSIVE TIE-2 RECEPTORS

Tie2 has recently been shown to promote and maintain skeletal muscle precursor cell quiescence (2). Ang-1, the activating ligand for Tie2, has been shown to transduce survival signals through integrins in mature myocytes (13). To investigate whether Ang-1 signaling through Tie2 might also play a role in muscle maturation and development, we initially examined Tie2 expression in mature skeletal muscle cells. By conventional RT-PCR we amplified a 3.4 kb band from both human skeletal myoblast and myotube RNA (Figure 1A), which was verified by sequencing as full-length Tie2. Tie2 mRNA expression in human and murine myoblasts was significantly lower than that in HUVECs (Figure 1B), although Tie2 protein was detectable by western blotting in human skeletal myoblasts, and treatment of these cells with Ang-1 induced phosphorylation of tyrosine residues 992 and 1102/1108, indicative of Tie2 activation (Figure 1C, D). Ad-Ang-1 adenovirus infection increased myoblast cell number and BrdU incorporation (Figure 2). Similarly, recombinant Ang-1 protein induced myoblast cell proliferation, but this effect was blocked in cells infected with an adenovirus encoding soluble Tie2 (ExTek) (Figure 2).

To investigate the pattern of Tie2 expression over the course of myoblast differentiation *in vitro*, primary mouse myoblasts were grown to 90% confluence and then switched to differentiation medium, and changes in Tie2 mRNA were quantified. Tie2 mRNA increased significantly by 48 hours, peaked 96 hours after

the onset of differentiation, and was maintained through 144 hours of differentiation (Figure 3A). These changes were confirmed in primary satellite cells isolated from Tie2Cre;mTmG transgenic mice, in which Tie2 promoter-driven Cre expression results in deletion of constitutive mTomato (mT) expression and expression of membrane-bound GFP (mG). Isolated myoblasts displayed no evidence of Tie2 promoter activity prior to the onset of differentiation, but by 96 hours of differentiation Tie2 expression was evident by the presence of GFP-expressing myotubes (Figure 3B). We further verified that Tie2 was expressed primarily in differentiated muscle cells. Immunoprecipitation of Tie2 from myoblasts, quiescent reserve cell (RC) populations, or fully differentiated myotubes (144hr DM) revealed detectable Tie2 expression only in myotubes (Figure 3C). Fluorescence microscopy of transverse sections of plantaris muscle from adult Tie2Cre;mTmG transgenic mice demonstrated heterogeneous GFP expression, consistent with scattered Tie2 expression at some point during the life of these muscle fibres (Figure 3D).

4.4.2 ANG-1 PROMOTES MYOBLAST DIFFERENTIATION AND PROLIFERATION Having establishing muscle cell-specific Tie2 expression, we next investigated whether myocyte-expressed Ang-1 could have direct effects on skeletal myoblasts (i.e., autocrine signaling), particularly on the expression of myogenic genes. Treatment of confluent mouse myoblasts for 24-hours with recombinant Ang-1 induced significant increases in p21, MyoD, myogenin, PGC1-α, and muscle creatine kinase (CK) mRNA expression (Figure 4A) and enhanced the expression of myosin heavy chain protein (Figure 4B), indicating that Ang-1 promotes differentiation of muscle precursor cells. Adenoviral over-expression of

Ang-1 in human skeletal myoblasts increased both mRNA (Figure 5A-C) and protein (Figure 5D) expression of the myogenic transcription factors MyoD and myogenin throughout differentiation. Similarly, Ang-1 over-expression triggered significant induction of myosin heavy chain mRNA and protein (Figure D) in human muscle cells. Collectively, these results demonstrate a role for Ang-1 in the promotion of both mouse and human muscle precursor cell differentiation. To confirm this finding, we measured the MyoD and myogenin regulatory axis of differentiation using a myogenin-specific reporter (Figure 6A) in mouse C2C12 (Figure 6B) and human skeletal myoblasts (Figure 6C) transfected with an Ang-1 expression plasmid in the absence or presence of MyoD. The presence of MyoD substantially induced myogenin promoter activity in both cell lines. In the absence of MyoD, overexpression of Ang-1 had no influence on the myogenin promoter. However, Ang-1 significantly increased myogenin promoter activity in the presence of MyoD, indicating that the induction of myogenin expression and the promotion of the muscle differentiation program by Ang-1 require the presence of MyoD (Figure6).

4.4.3 ANG-1 SIGNALS THROUGH MAP KINASES AND AKT/mTOR IN SKELETAL MUSCLE CELLS

We next examined signaling pathways induced by Ang-1 in skeletal muscle cells. Exposure of human skeletal myoblasts to Ang-1 triggered transient but significant increases in phosphorylation of paxillin, FAK1, ERK1/2, p38, and SAPK (Figure 7A). Phosphorylation of these proteins peaked at 5 to 15 min after the addition of Ang-1 and returned to baseline or even below baseline values after 60 min of Ang-1 exposure (Figure 7B). In addition, Ang-1 exposure elicited marked increases in

phosphorylation of AKT, mTOR, P70S6 kinase, and 4E-BP1 (Figure 7C). Ang-1 treatment also increased the phosphorylation of the AP-1 subunit of c-Jun (Figure 7D), collectively suggesting that Ang-1 activates myocyte signaling involved in cell proliferation, survival, motility, and stress responsiveness.

Based on Ang-1's effects on myoblast signaling pathways, we also examined the effects of Ang-1 on myoblast survival and migration. Ang-1 significantly attenuated the rise in cytotoxicity and caspase-3/7 activity associated with serum starvation in culture (Figure 8) and induced a significant increase in the rate of myoblast migration in an *in vitro* scratch assay (Figure 8). These effects were specific to Ang-1, as Ang-2 did not induce similar effects (Figure 8). Together, these data support a role for Ang-1 in promoting cellular responses necessary for myoblast fusion during skeletal muscle plasticity.

4.4.4 ANG-1 ENHANCES SKELETAL MUSCLE REGENERATION FOLLOWING INJURY

Based on the observed effects of Ang-1/Tie2 signaling in myocytes, we next examined the effects of Ang-1 on regeneration following cardiotoxin (*Naja nigricollis* venom) injection into the tibialis anterior (TA) muscle in mice. Administration of Ad-Ang-1 significantly reduced myofibre injury, as measured by total fibre number (Figure 9A), and enhanced the number of myofibres with two or three central myonuclei (Figure 9B), consistent with an effect on myofibre regeneration. No significant difference in fibre cross sectional area was observed between muscles treated with Ad-GFP or Ad-Ang-1 (Figure 9C), indicating that Ang-1 expression did not elicit myofibre hypertrophy. Contractility of injured TA muscles

treated with Ad-Ang-1 completely recovered by 10 days after cardiotoxin injection, while injured muscles treated with Ad-GFP remained impaired (Figure 10). Injection of Ad-Ang-1 also resulted in a significantly greater number of fibres expressing embryonic myosin heavy chain, a marker of regenerating muscle fibres (Figure 11). Capillary density was also significantly greater in Ad-Ang-1-treated muscles compared to those treated with Ad-GFP (0.90±0.04 vs. 0.62±0.03 capillaries/muscle fibre, $P<0.05$). RNA expression of the endothelial cell markers PECAM-1 and Tie1 increased following Ad-Ang-1 treatment (Figure 12), further indicating that Ang-1 augmented vascular density in addition to promoting muscle regeneration.

We next examined the patterns of angiopoietin expression in muscle during regeneration. By immunostaining, Ang-1 protein was detected mainly in satellite cells in intact and regenerated muscle (Figure 13A,C,F). Ang-1 staining was not detected in nerve fibres (Figure 13B) but was evident in blood vessels (Figure 13). Ang-2 protein was detected in blood vessels as well as in un-injured and regenerated skeletal muscle fibres (Figure 14A,B,D) and inflammatory cells (Figure 14C). Ang-1 mRNA in whole muscle did not change over two weeks following cardiotoxin-induced injury (Figure 15). In contrast, Ang-2 mRNA expression increased significantly within 1 day after injury and remained modestly but significantly elevated over the subsequent 2 weeks (Figure 15). Tie2 mRNA levels rose significantly after 1 and 14 days of cardiotoxin injection ($p<0.05$ compared to un-injured muscle). VEGF expression declined significantly but transiently after 1 and 3 days of injury and then returned to levels similar to those of un-injured muscle (Figure 15). Northern blot analysis of human skeletal myoblasts demonstrated a single Ang-1 mRNA transcript but failed to

detect an Ang-2 transcript in myoblasts, although Ang-2 was abundantly expressed in HUVECs (Figure 16A). Real-time PCR analysis confirmed that Ang-1 is the most abundantly expressed angiopoietin in primary human and murine myoblasts, while Ang-2 was barely detectable (Figure 16B) and Ang-4 was undetectable by either PCR or Northern blotting (not shown). Immunoblotting confirmed the presence of Ang-1 protein in myoblast lysates and cell culture medium (Figure 16C). Ang-1 and Ang-2 mRNA expression increased during differentiation of human myoblasts to myotubes, peaking on day 3 of differentiation and subsequently declining (Figure 16D), confirming that skeletal myoblasts produce and secrete angiopoietins and suggesting that muscle cells may contribute to increased Ang-1 and Ang-2 mRNA levels during muscle regeneration. Collectively, these data demonstrate that Ang-1 is expressed by skeletal muscle cells, and they suggest Ang-1 may contribute to skeletal muscle regeneration through both paracrine signaling, by activating endothelial cells, and autocrine signaling, acting directly on Tie2-expressing myotubes.

To confirm *in vivo* expression of Tie2 specifically in regenerating skeletal muscle cells, we performed immunofluorescence microscopy on regenerating muscle following cardiotoxin injection. These studies revealed Tie2 staining not only in capillaries and infiltrating mononuclear cells but also in regenerating myofibres, evidenced by the presence of centralized myonuclei, during the initial week (CTX d7) of regeneration (Figure 17), demonstrating wide heterogeneity in cellular Tie2 expression. At day 14 of recovery (CTX d14), following resolution of the inflammatory process, Tie2 staining was still detectable in regenerating myofibres

independent of endothelial CD31 staining (Figure 17), demonstrating specific expression of Tie2 in differentiating myocytes.

4.4.5 MYOCYTE DIFFERENTIATION IS TIE2-DEPENDENT IN THE ABSENCE OF EXOGENOUS ANG-1

To examine whether Tie2 is required for Ang-1's ability to drive muscle differentiation, we used recombinant retrovirus to generate lines of primary mouse myoblasts expressing control ($Tie2^{Scr}$) or Tie2-specific shRNAs ($Tie2^{low}$) (Figure 18A). $Tie2^{low}$ myoblasts appeared normal by morphological analysis up to 48-hours after the onset of differentiation but demonstrated increased numbers of immature myotubes at later stages of differentiation (Figure 18B). Tie2 silencing did not alter the early transcriptional differentiation program at 48hrs but significantly attenuated expression of p21, MyoD, and myogenin mRNA at 96- and 144-hours of differentiation compared to that in control $Tie2^{Scr}$ cells (Figure 19), demonstrating that Tie2 is required for sustained activation of the muscle transcriptional differentiation program. However, treatment of $Tie2^{low}$ myoblasts with recombinant Ang-1 rescued the deficits in p21, MyoD, and myogenin mRNA expression at later stages of differentiation (96 and 144 hours) (Figure 19). These results demonstrate that exogenous Ang-1 is sufficient to drive myocyte expression of p21, MyoD, and myogenin mRNA even in the relative absence of Tie2 expression.

To identify the specific genetic program activated by Ang-1 in skeletal muscle cells, the transcriptional profile of human skeletal myoblasts expressing Ang-1 was analyzed using an Illumina microarray. Expression of 22 genes was significantly upregulated by Ang-1 in skeletal myoblasts compared to control-treated cells (Table

2). The largest relative increases were in TEL2 (regulator of cell cycle) and NRP1 (receptor for VEGF family of proteins). Ang-1 also induced the expression of several other genes involved in angiogenesis, proliferation, adhesion, and cell-cell signaling, including fibroblast growth factor 4, LEP (leptin), Angiogenin, Protocadherin 1, and the CCL2 chemokine (Table 2). Ang-1 significantly downregulated the expression of 31 genes, including those involved in inhibition of angiogenesis (SERPINB2 and SERPINC1), protein folding (Heat Shock 70kDa), transcription (Spanx transcription factors, FOS), nitrosative stress (inducible nitric oxide synthase), and two regulators of angiogenesis (Angiopoietin-like 4 and hepatocyte growth factor) (Table 2). Intriguingly, HGF also promotes myoblast quiescence, thus the downregulation of this factor is consistent with a role for Ang-1 in promoting myoblast differentiation.

Given the relative absence of Tie2 expression during the early phase of differentiation (initial 48-hours) in our *in vitro* model, we hypothesized that Tie2 is not necessary early in the induction of differentiation (<48hrs) but that its expression is required later for sustained myocyte differentiation (i.e., beyond 48hrs). To test this hypothesis, we first rescued mouse $Tie2^{low}$ myoblast receptor expression stably using human full-length Tie2 retrovirus and secondary antibiotic selection ($Tie2^{low+hTie2\text{-}RV}$) and allowed these cells to reach confluence and differentiate for the full 144hour time-course. $Tie2^{low}$ myoblasts were transiently transfected at 24 and then 48-hours of differentiation with GFP ($Tie2^{low+GFP}$), full-length human Tie2 ($Tie2^{low+hFL}$), or a truncated mutant human Tie2 lacking the cytoplasmic kinase domain ($Tie2^{low+hTM}$) and returned to differentiation medium for the remaining 48-144hrs of differentiation (Figure 20). Transfection of $Tie2^{low+hFL}$ after the onset of

differentiation partially rescued both the morphological, gene expression (p21, MyoD, and myogenin), and contractile protein (Figure 20) deficits observed in $Tie2^{low}$ cells, demonstrating a temporal requirement for Tie2 expression in the maturation of skeletal myotubes *in vitro*. Furthermore, there is a functional requirement for Tie2 catalytic activity in this process, as rescue with the truncated mutant failed to restore myotube differentiation (Figure 20).

4.5 DISCUSSION

In this report we demonstrate the novel finding that Ang-1/Tie2, a ligand-receptor pair traditionally linked to regulation of endothelial cell signaling and vascular growth plays an essential role in skeletal myoblast differentiation, both in vivo and in vitro, in the absence of paracrine signaling from the endothelium. Ang-1 has been shown to transduce signals in differentiated myotubes through integrins (13), and Ang-1/Tie2 signaling has been implicated in satellite cell quiescence (2). However, our current results establish a novel role for Ang-1 and Tie2 in regulation of the myogenic differentiation program during skeletal muscle regeneration following injury. In this setting, the Ang-1-mediated increase in myofibre differentiation translated into enhanced muscle force development in vivo. Furthermore, Ang-1's effects on myoblast differentiation in vitro were dependent on Tie2, which was expressed in differentiating myoblasts in a precise temporal pattern.

The angiopoietins are a family of growth factors (including Ang-1-4) that bind with varying affinity to members of the Tie family of receptor tyrosine kinases (Tie1, Tie2) and are critical for the formation and maintenance of blood vessels (17, 39). Skeletal muscle cells respond to angiopoietins both as myoblasts and differentiated myotubes in a manner previously shown to be at least partly integrin receptor-dependent (13). Mature murine myotubes display much greater affinity for Ang-1 in vitro than do undifferentiated myoblasts (13). Although this difference was originally attributed to increased integrin subunit expression with myotube maturation, exponentially higher expression of Tie2 with myocyte differentiation, as

observed in our studies and reported by others (2), also appears to be responsible for this increased affinity.

We found that myocyte-expressed Tie2 was activated by Ang-1 in skeletal muscle cell cultures *in vitro*, and Ang-1 induced numerous cellular responses in these cells, including proliferation, migration, and survival, similar to its effects on endothelial cells. Somewhat surprisingly, Ang-1 also induced a strong myogenic signaling effect in skeletal muscle both *in vitro* and *in vivo*. Ang-1 promoted expression of myogenic regulatory factors and myosin heavy chain and enhanced muscle differentiation by promoting myogenin transcription *in vitro*. *In vivo*, Ang-1 overexpression enhanced muscle myofibre regeneration and rapidly restored muscle contractile function after injury. Interestingly, our results demonstrated that centralized nuclei in regenerating myofibres expressed Tie2, suggesting a distinct and previously unrecognized role for Tie2 in muscle differentiation and regeneration. Consistent with these findings, stable knockdown of Tie2 *in* vitro using shRNA ($Tie2^{low}$) resulted in primary satellite cell cultures that were retarded in their ability to fully differentiate. The primary morphological and myogenic regulatory factor (MyoD, myogenin) deficits in these cells were observed after 48-hours of differentiation, at a time concomitant with increased endogenous expression of Tie2 and suggesting a direct role for Tie2 signaling in sustained muscle differentiation. Our results also demonstrated a time-dependent requirement for catalytically-active Tie2 in order for full maturation to occur in the absence of exogenous Ang-1, as transient expression of human Tie2 after 48-hours of differentiation partially rescued the $Tie2^{low}$ phenotype. Importantly, this effect required Tie2 kinase activity, as a

truncated mutant of the receptor lacking the cytosolic kinase domain was unable to rescue the defective phenotype. Interestingly, Ang-1 also functioned as a potent myogenic signaling molecule in a Tie2-independent manner, as high concentrations of exogenous Ang-1 were able to rescue deficits in sustained muscle differentiation in $Tie2^{low}$ cells *in vitro*. Altogether, these observations show that Tie2 expression and activity are required for signaling muscle differentiation processes and that Ang-1 functions through complementary Tie2-dependent and -independent pathways to promote skeletal muscle precursor cell regenerative responses.

Skeletal muscle recovery following injury is regulated by a highly coordinated gene expression program that drives muscle precursor cell proliferation and differentiation (6, 54). In the cardiotoxin injury model, activation of satellite cell proliferation begins as early as 6-hours after injury and transitions to a sustained differentiation program after 5-6 days, which coincides with reconstitution of the fascicular fibre architecture and appearance of satellite cells as differentiating centralized nuclei (21). The myogenic transcriptional program induced in regenerating muscle relies heavily on the basic helix-loop-helix (bHLH) transcription factors, including MyoD, Myf5, MRF4, and myogenin (21), and the cyclin-dependent kinase inhibitor p21 (22). Notably, similar transcriptional programs drive both skeletal muscle satellite cell proliferation and differentiation *in vivo* and the maturation of isolated myoblasts *in vitro*. Our *in vivo* studies demonstrated that Ang-1 drives the differentiation process by enhancing expression of embryonic myosin heavy chain following cardiotoxin-induced injury. Because Tie2 is expressed predominantly on endothelial cells, it is possible that the effects of Ang-1 *in* vivo

were due to paracrine signaling resulting from Ang-1's effects on endothelial cells. However, similar effects of Ang-1 on the myogenic differentiation program were noted in isolated skeletal myocytes *in vitro*, ruling out a contribution from endothelial Tie2 signaling to differentiation. Moreover, Tie2 protein expression was demonstrated around centralized myonuclei at a time point during regeneration coincident with satellite cell incorporation and differentiation and consistent with a distinct role for Tie2 in the regulation of this phase of satellite cell differentiation.

Our findings differ from the recent observations of Abou-Khalil *et al.* (2), which suggested a specific role for Ang-1/Tie2 in maintenance of the reserve cell population of satellite cells. Whereas they found that Tie2 expression was highest in quiescent satellite cells, we observed a significant increase in Tie2 mRNA expression with progressive differentiation, and Tie2 protein was undetectable *in vitro* until myotubes had fully differentiated (6 days). It should be noted, however, that Abou-Khalil *et al.* used flow cytometry to select for $Tie2^{+}$ satellite cells, an approach that is not technically feasible with differentiated myotubes. This approach likely sorted a rare population of myoblasts with relatively high Tie2 levels and in which Tie2 regulates quiescence. In contrast, our results support a role for Tie2 in myoblast differentiation, as loss of Tie2 in myoblasts prevented full differentiation, with deficient transcriptional expression of the muscle differentiation regulators p21, MyoD, and myogenin during myotube formation. These defects could be rescued by re-expression of Tie2 in the later phases of the differentiation process (i.e., after 48 hours). Strikingly, constitutive re-expression of Tie2 in myoblasts prevented

differentiation, arguing that Tie2 may regulate both quiescence and differentiation in a time- and context-dependent manner.

Another notable difference in our findings from those of Abou-Khalil *et al.* (2) was the strong pro-regenerative/differentiation effect of exogenous Ang-1 both *in vivo* and *in vitro*. The timing of Tie2 expression in regenerating skeletal muscle *in vivo* and developing myotubes *in vitro* suggests that it may function after myoblast fusion, a time frame characterized by sarcomeric assembly in the mature myotube/myofibre. In our *in vivo* study, Ang-1 was delivered after the initial period of recovery (after regeneration day 4), a time point that matches the onset of Tie2 expression during muscle recovery. During this period of postnatal muscle differentiation, expression of myosin, M-line proteins, and serum response factor (SRF)-regulated striated α-actin occurs (29, 49), indicating functional organization of myotube sarcomeric structure. SRF is necessary for late skeletal muscle growth/maturation *in vivo* and *in vitro* and is believed to function in concert with myocardin-related transcription factors (MRTFs) and KLF3 at CArG motifs at sequentially later stages of muscle maturation involving myoblast proliferation and fusion (23, 29, 48) A specific role has been established *in vitro* for SRF in β-integrin-mediated RhoA signaling and regulation of α-actin expression in the late stages of myocyte maturation (49). Notably, Ang-1 has been shown to signal via β1 integrins in both skeletal muscle and endothelial cells (9, 13), thus it is possible that the Ang-1/Tie2 signaling axis functions as a complementary pathway to regulate SRF transcriptional activity and sarcomeric organization during the later stages of myocyte differentiation. Our results establish a role for Tie2 kinase activity in MyHC protein

expression during sustained differentiation. However, it is not yet known whether Tie2 is required for MEF2C- and SRF-induced transcriptional expression of myofibrillar proteins and subsequent organization of the mature sarcomere, and such findings would firmly establish Ang-1 as a potent anabolic signaling molecule in muscle biology.

In support of the recent findings of Abou-Khalil *et al.*, Ang-1 has been shown previously to regulate hematopoietic stem cell (HSC) quiescence in part by promoting adhesion of Tie2-expressing HSCs to endosteal osteoblasts (3). Although it is unclear what spatial and/or contextual cues contribute to Ang-1's effects in muscle, the proximity of satellite cells to a variety of different cellular or extracellular matrix components may dictate whether Ang-1 induces quiescence or differentiation. Moreover, local Ang-1 concentration may be an important aspect of its ultimate effect on muscle precursor cell fate. Hepatocyte growth factor (HGF) is known to have concentration-dependent effects on satellite cell quiescence or differentiation, at least *in vitro* (44). Although Ang-1 is a secreted growth factor, it binds preferentially in the extracellular matrix (51), thus effective concentrations of Ang-1 in specific intercellular locations may be higher than expected based on tissue mRNA expression and may facilitate satellite cell quiescence or differentiation.

In endothelial cells, Ang-1 induces AKT activation and cell quiescence as well as co-localization of $\alpha v\beta 3$ integrin complex with Tie2, however Ang-2 recruitment of the integrin subunit results in its internalization and degradation (45). Our results demonstrate that expression of Ang-1 and Ang-2 is strongly induced at the onset of differentiation in skeletal myoblasts, which may provide a mechanism for

degradation of pre-maturation integrin complexes concomitant with the increased expression of β1D variants in mature myofibres/myotubes. Furthermore, overexpression of Ang-1 or treatment of confluent myoblasts with exogenous Ang-1 resulted in the robust initiation of the muscle differentiation program and activation of a variety of signaling pathways, including focal adhesion kinase, protein synthetic signaling, ERK1/2, and the immediate early c-Jun protein, prior to the onset of Tie2 expression. These results demonstrate the potency of Ang-1 as a myogenic stimulator, a finding that was confirmed by the ability of exogenous Ang-1 to rescue the morphological and transcriptional deficits observed in $Tie2^{low}$ differentiating myoblasts *in vitro* and to significantly accelerate the timeline of muscle regeneration *in vivo*. Ang-1 has been shown to induce skeletal muscle mitochondrial biogenesis in diabetic mice (53), and in the present study Ang-1 increased expression of PGC1-α and creatine kinase in differentiating myoblasts, consistent with an important role for Ang-1 in regulation of bioenergetic metabolism in mature myofibres/myotubes. These studies suggest that, regardless of whether Ang-1 acts through Tie2 or integrins, exogenous Ang-1 have potential therapeutic utility for skeletal myopathies in addition to its potential for improving vascular stabilization.

In conclusion, we propose a novel model in which Tie2 is expressed not only in endothelial cells but also in mature skeletal muscle myofibres, where it plays a requisite role in the initiation of sustained differentiation in muscle precursor cells. Furthermore, the Tie2 ligand Ang-1 signals in skeletal muscle through complementary pathways that are both Tie2-dependent and -independent to enhance myoblast fusion, differentiation and recovery from injury.

4.6 ACKNOWLEDGMENT:

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4.8 FIGURES

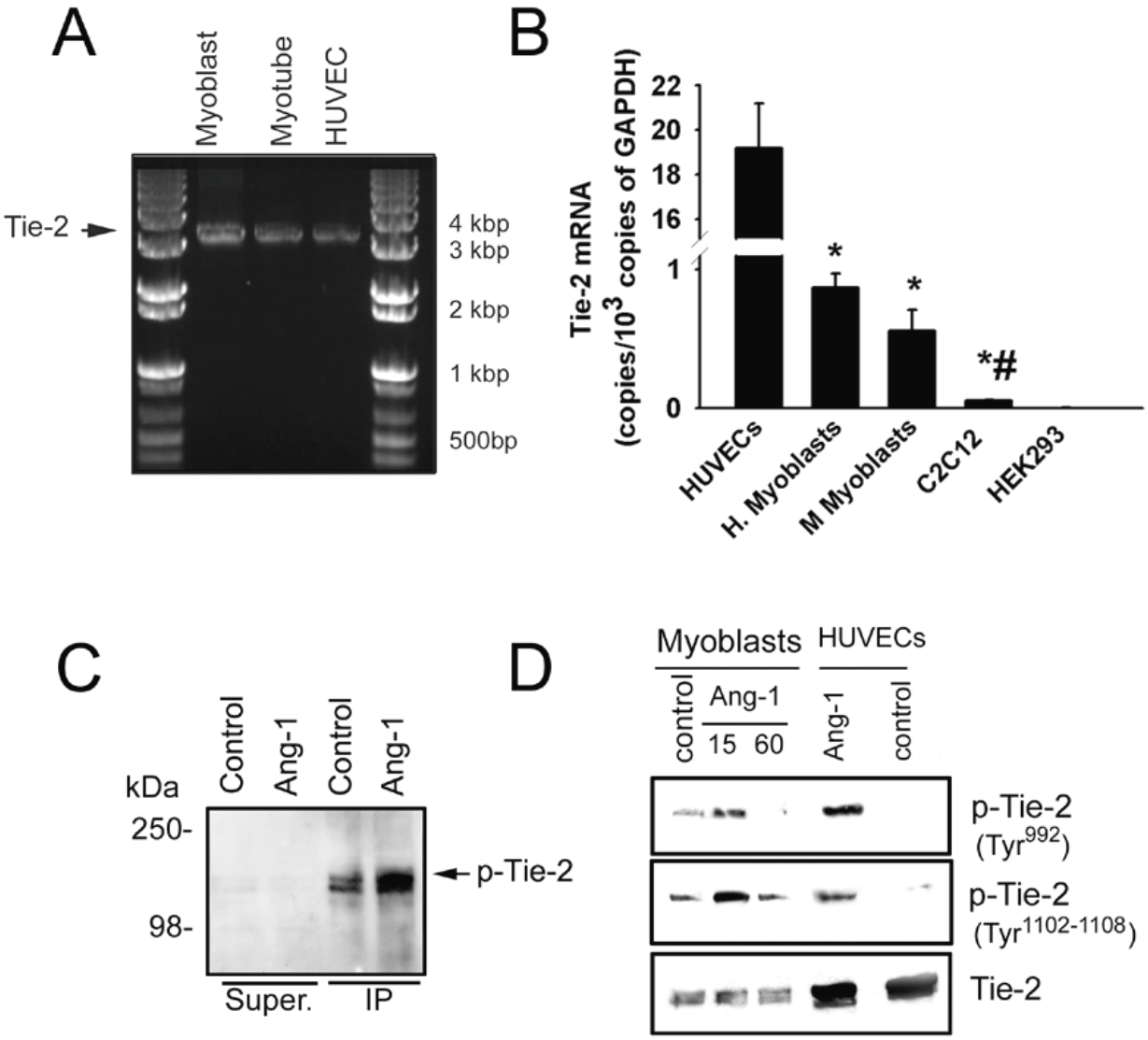

FIGURE 1

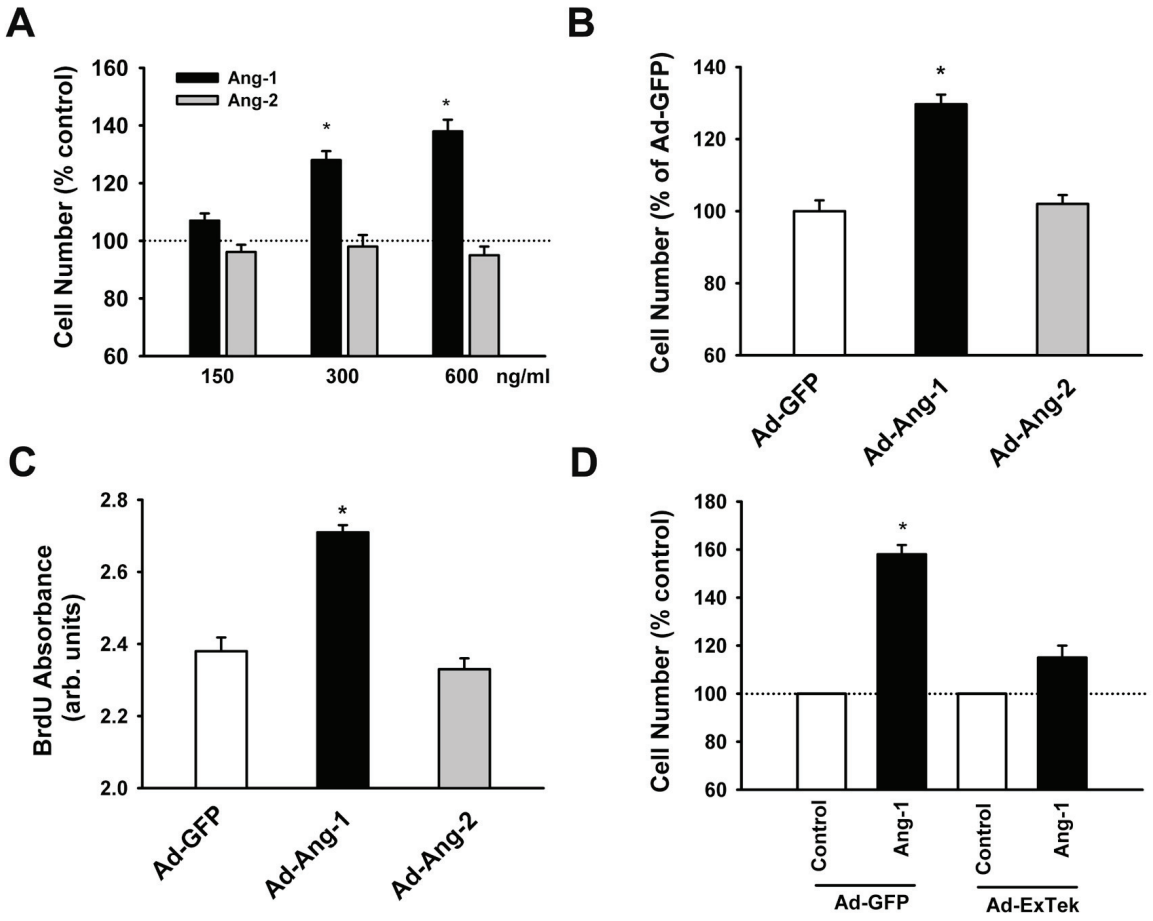
A
Cell Number (% control)
160
140
120
100
80
60
Ang-1
Ang-2
*
*
150
300
600
ng/ml
B
Cell Number (% of Ad-GFP)
140
120
100
80
60
*
Ad-GFP
Ad-Ang-1
Ad-Ang-2
C
BrdU Absorbance (arb. units)
2.8
2.6
2.4
2.2
2.0
*
Ad-GFP
Ad-Ang-1
Ad-Ang-2
D
Cell Number (% control)
180
160
140
120
100
80
60
*
Control
Ang-1
Control
Ang-1
Ad-GFP
Ad-ExTek

FIGURE 2

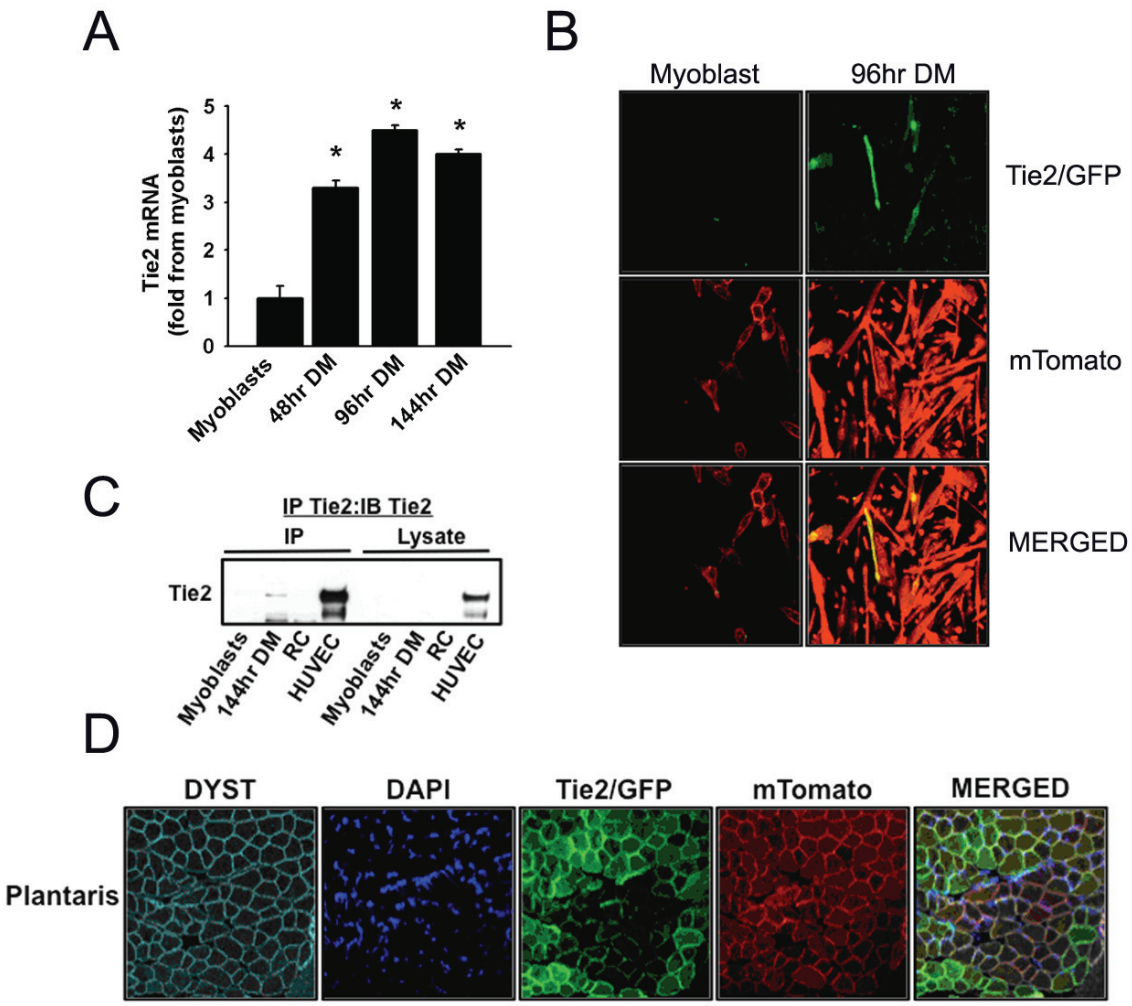
A
Tie2 mRNA (fold from myoblasts)
0
1
2
3
4
5
*
*
*
Myoblasts
48hr DM
96hr DM
144hr DM
B
Myoblast
96hr DM
Tie2/GFP
mTomato
MERGED
C
IP Tie2:IB Tie2
IP
Lysate
Tie2
Myoblasts
144hr DM
RC
HUVEC
Myoblasts
144hr DM
RC
HUVEC
D
DYST
DAPI
Tie2/GFP
mTomato
MERGED
Plantaris

FIGURE 3

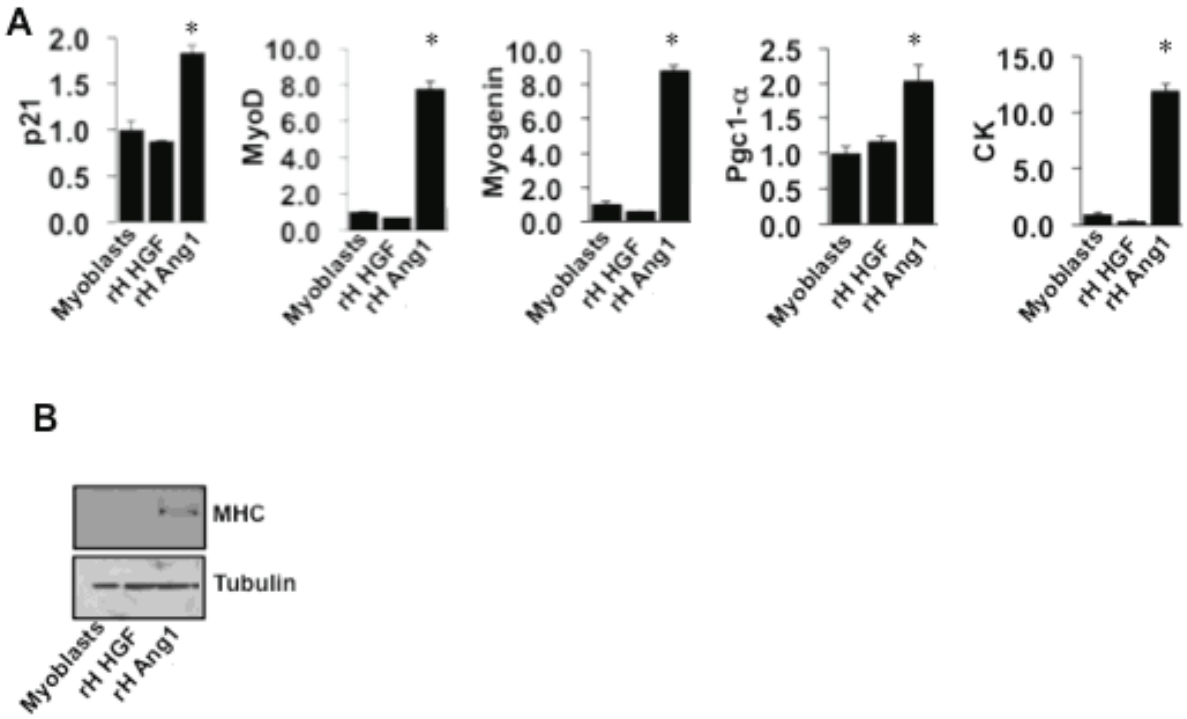
A
p21
MyoD
Myogenin
Pgc1-α
CK
Myoblasts
rH HGF
rH Ang1
B
MHC
Tubulin

FIGURE 4

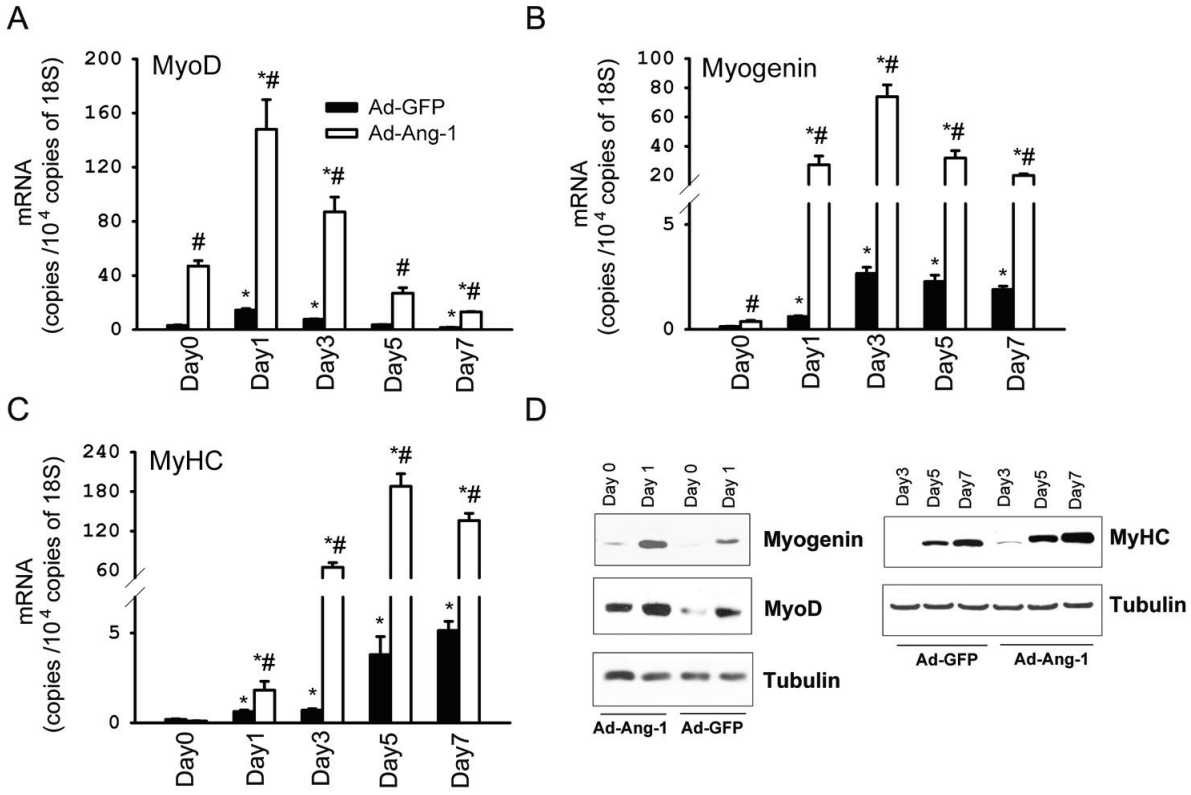
A
MyoD
mRNA (copies /10^4 copies of 18S)
Ad-GFP
Ad-Ang-1
Day0
Day1
Day3
Day5
Day7
B
Myogenin
C
MyHC
D
Day 0
Day 1
Myogenin
MyoD
Tubulin
Ad-Ang-1
Ad-GFP
Day3
Day5
Day7
MyHC

FIGURE 5

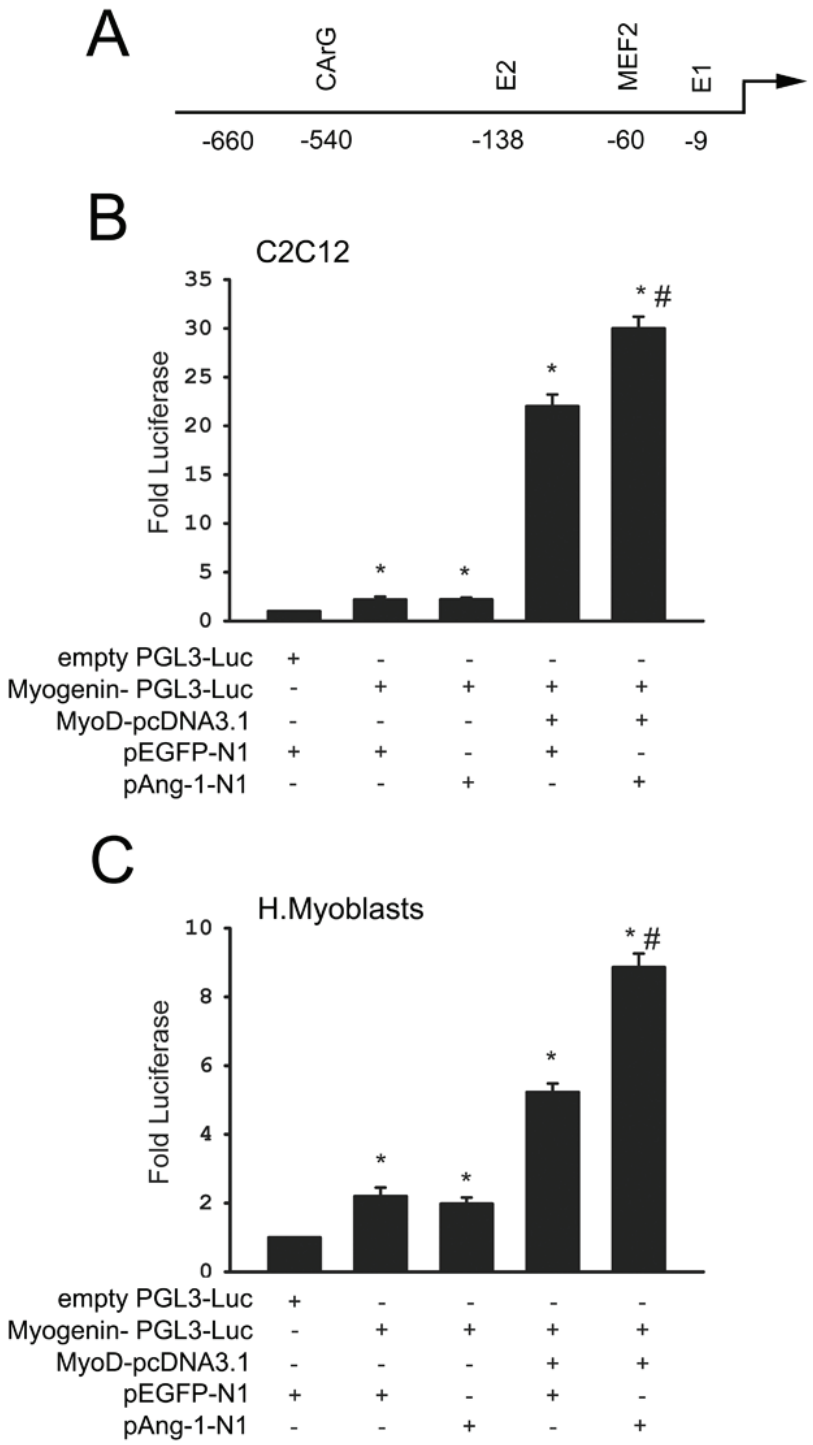
A
CArG
E2
MEF2
E1
-660
-540
-138
-60
-9
B
C2C12
Fold Luciferase
35
30
25
20
15
10
5
0
* #
*
*
*
*
empty PGL3-Luc + - - - -
Myogenin- PGL3-Luc - + + + +
MyoD-pcDNA3.1 - - - + +
pEGFP-N1 + + - + -
pAng-1-N1 - - + - +
C
H.Myoblasts
Fold Luciferase
10
8
6
4
2
0
* #
*
*
*
*
empty PGL3-Luc + - - - -
Myogenin- PGL3-Luc - + + + +
MyoD-pcDNA3.1 - - - + +
pEGFP-N1 + + - + -
pAng-1-N1 - - + - +

FIGURE 6

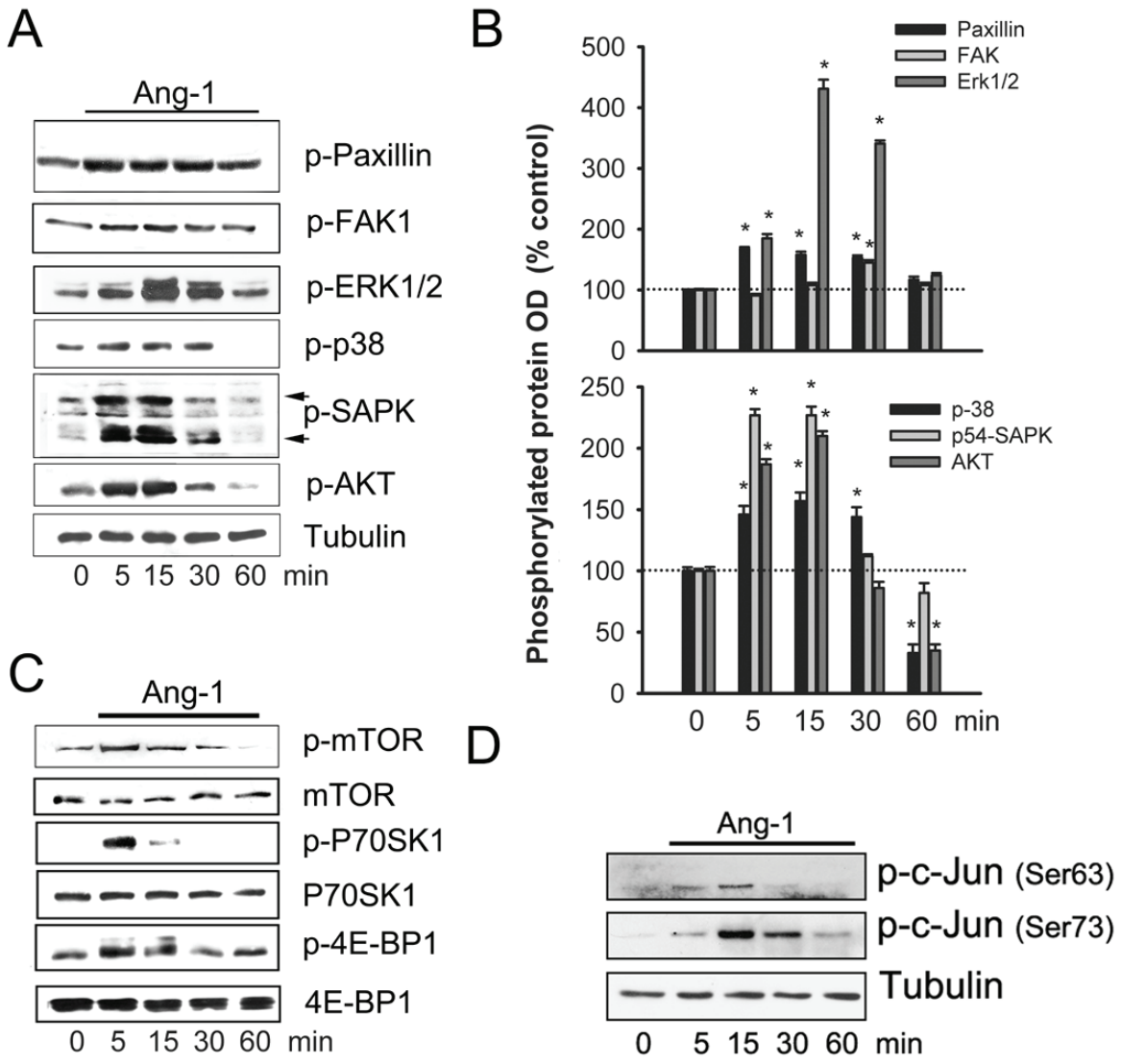
A
Ang-1
p-Paxillin
p-FAK1
p-ERK1/2
p-p38
p-SAPK
p-AKT
Tubulin
0 5 15 30 60 min
B
Paxillin
FAK
Erk1/2
Phosphorylated protein OD (% control)
500
400
300
200
100
0
p-38
p54-SAPK
AKT
250
200
150
100
50
0
0 5 15 30 60 min
C
Ang-1
p-mTOR
mTOR
p-P70SK1
P70SK1
p-4E-BP1
4E-BP1
0 5 15 30 60 min
D
Ang-1
p-c-Jun (Ser63)
p-c-Jun (Ser73)
Tubulin
0 5 15 30 60 min

FIGURE 7

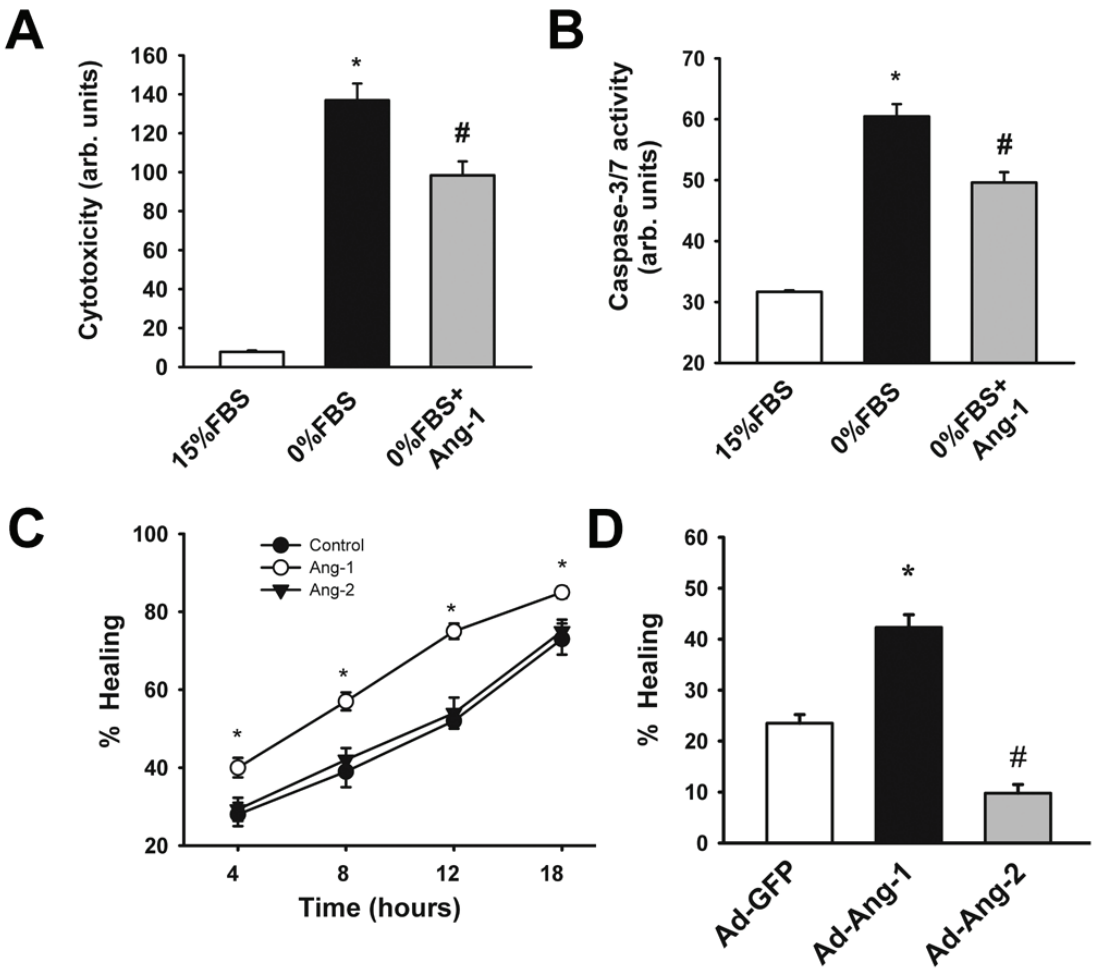
A
Cytotoxicity (arb. units)
160
140
120
100
80
60
40
20
0
*
#
15%FBS
0%FBS
0%FBS+ Ang-1
B
Caspase-3/7 activity (arb. units)
70
60
50
40
30
20
*
#
15%FBS
0%FBS
0%FBS+ Ang-1
C
% Healing
100
80
60
40
20
Control
Ang-1
Ang-2
*
4
8
12
18
Time (hours)
D
% Healing
60
50
40
30
20
10
0
*
#
Ad-GFP
Ad-Ang-1
Ad-Ang-2

FIGURE 8

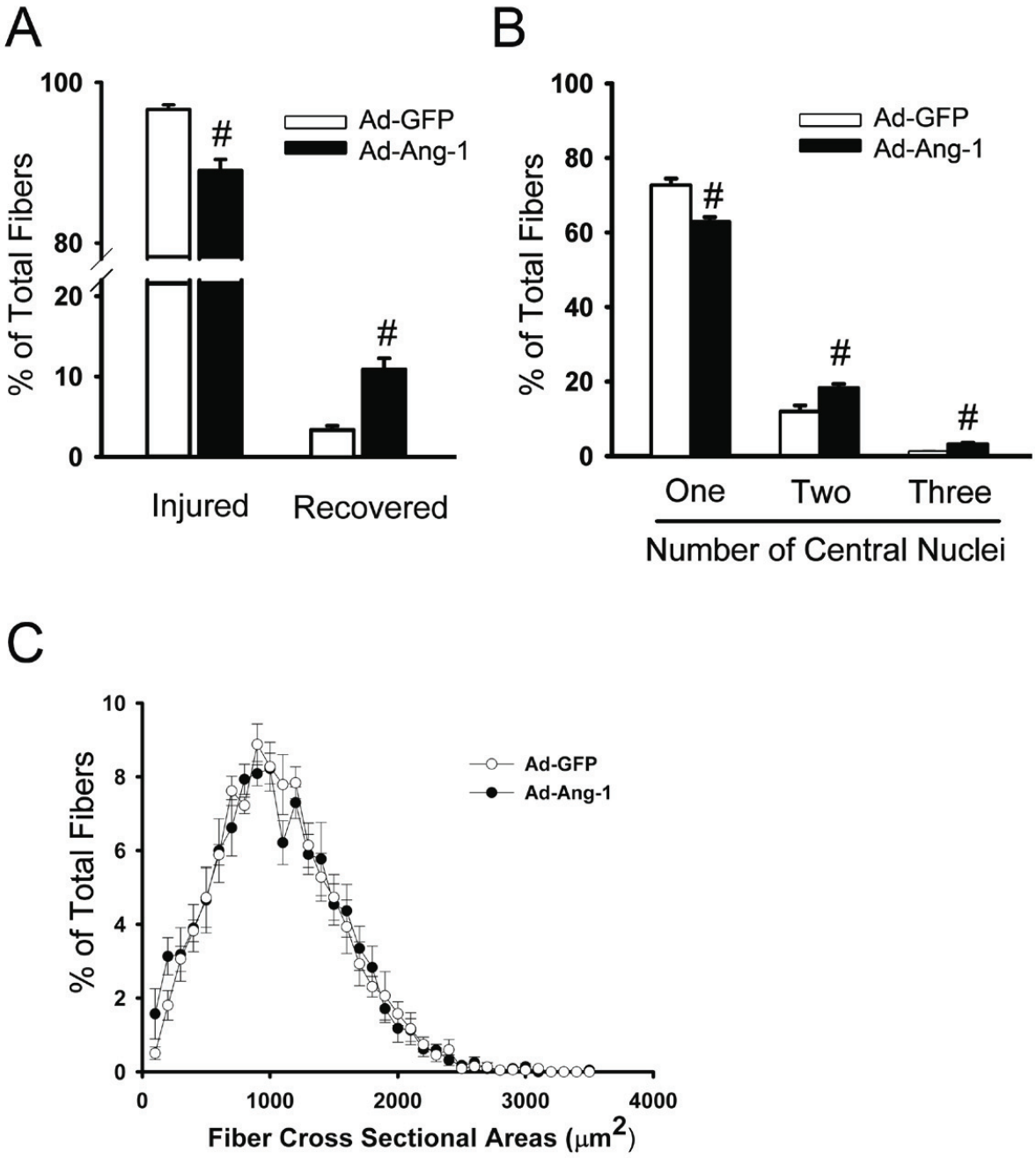
A
100
80
20
10
0
% of Total Fibers
#
#
Ad-GFP
Ad-Ang-1
Injured
Recovered
B
100
80
60
40
20
0
% of Total Fibers
#
#
#
Ad-GFP
Ad-Ang-1
One
Two
Three
Number of Central Nuclei
C
10
8
6
4
2
0
% of Total Fibers
Ad-GFP
Ad-Ang-1
0
1000
2000
3000
4000
Fiber Cross Sectional Areas (μm2)

FIGURE 9

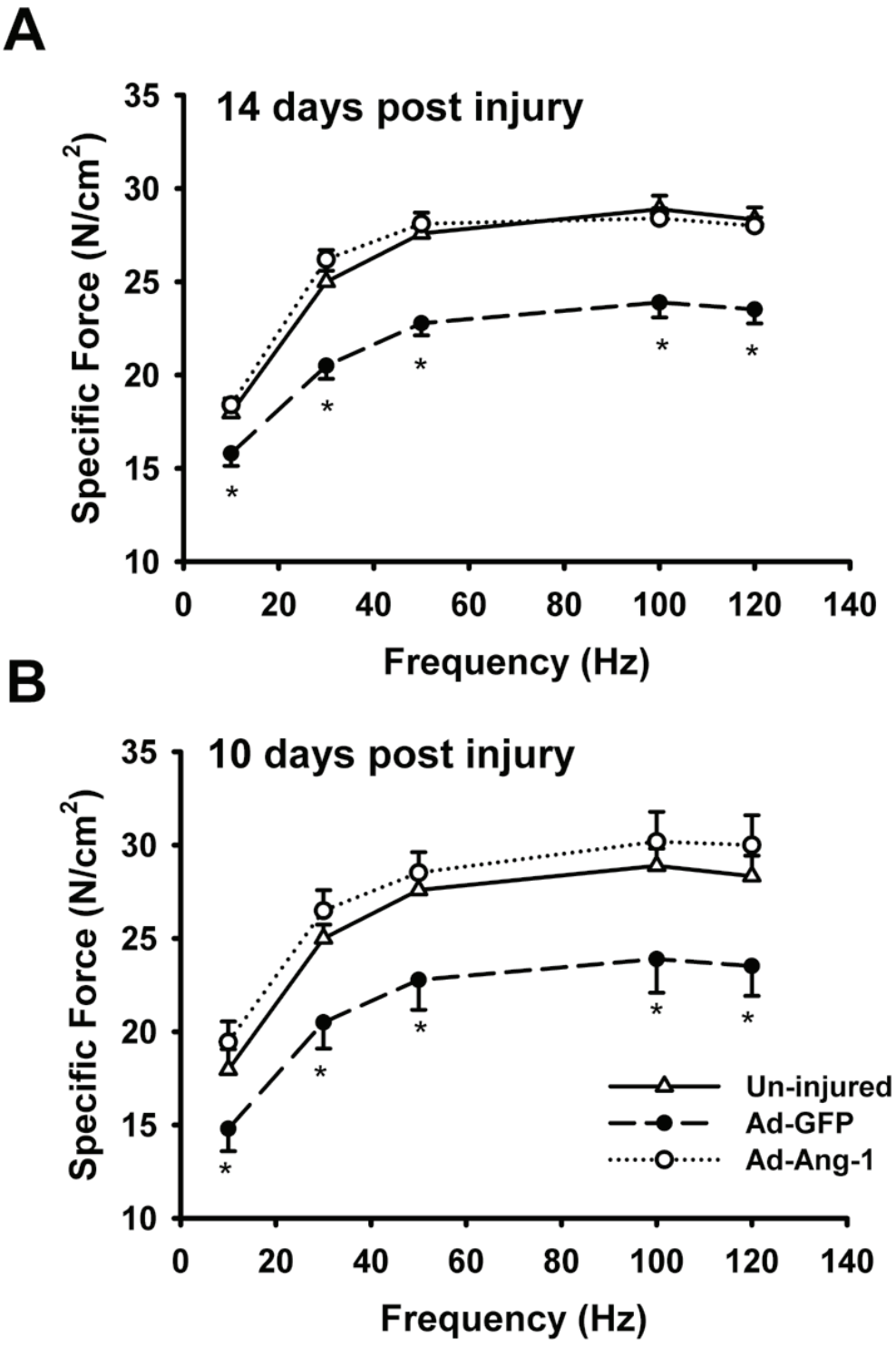
A
14 days post injury
Specific Force (N/cm2)
35
30
25
20
15
10
0
20
40
60
80
100
120
140
Frequency (Hz)
B
10 days post injury
Specific Force (N/cm2)
Un-injured
Ad-GFP
Ad-Ang-1
Frequency (Hz)

FIGURE 10

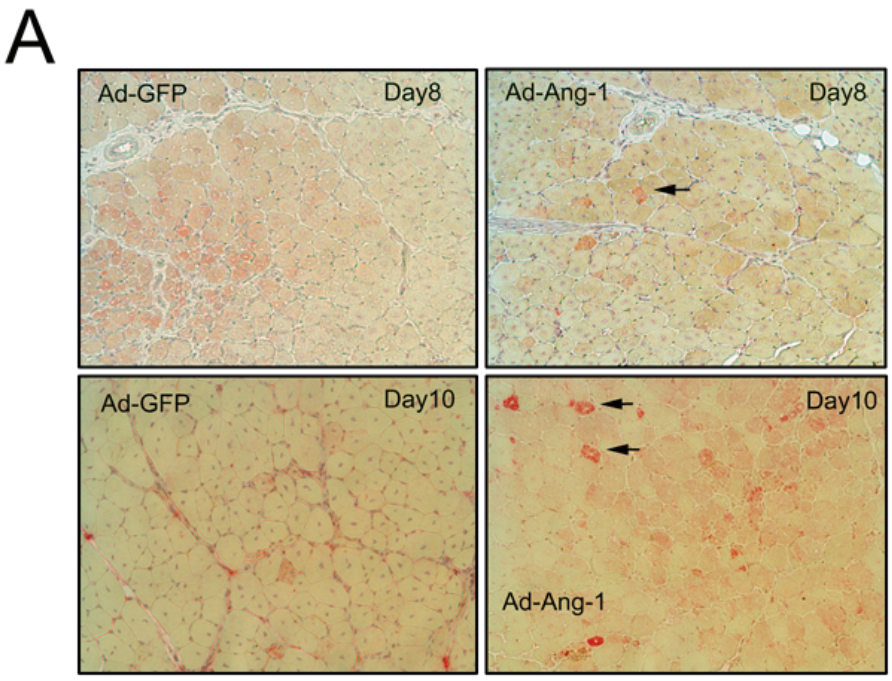
A
Ad-GFP
Day8
Ad-Ang-1
Day8
Ad-GFP
Day10
Day10
Ad-Ang-1
B
Fibers positive for e-MyHC
(% of total fiber number)
50
40
30
20
10
0
Ad-GFP
Ad-Ang-1
*
*#
*
*#
Day6
Day8
Day10

FIGURE 11

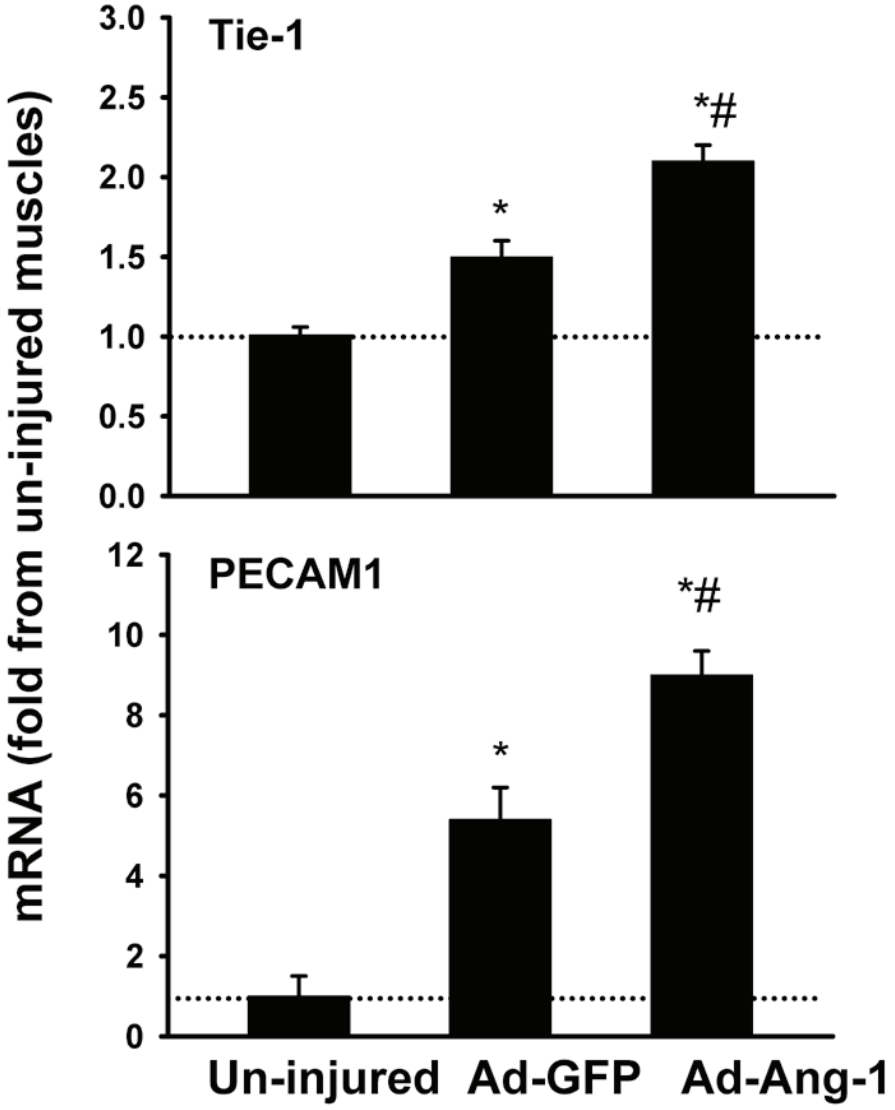
Tie-1
PECAM1
mRNA (fold from un-injured muscles)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
12
10
8
6
4
2
0
*
*#
Un-injured
Ad-GFP
Ad-Ang-1

FIGURE 12

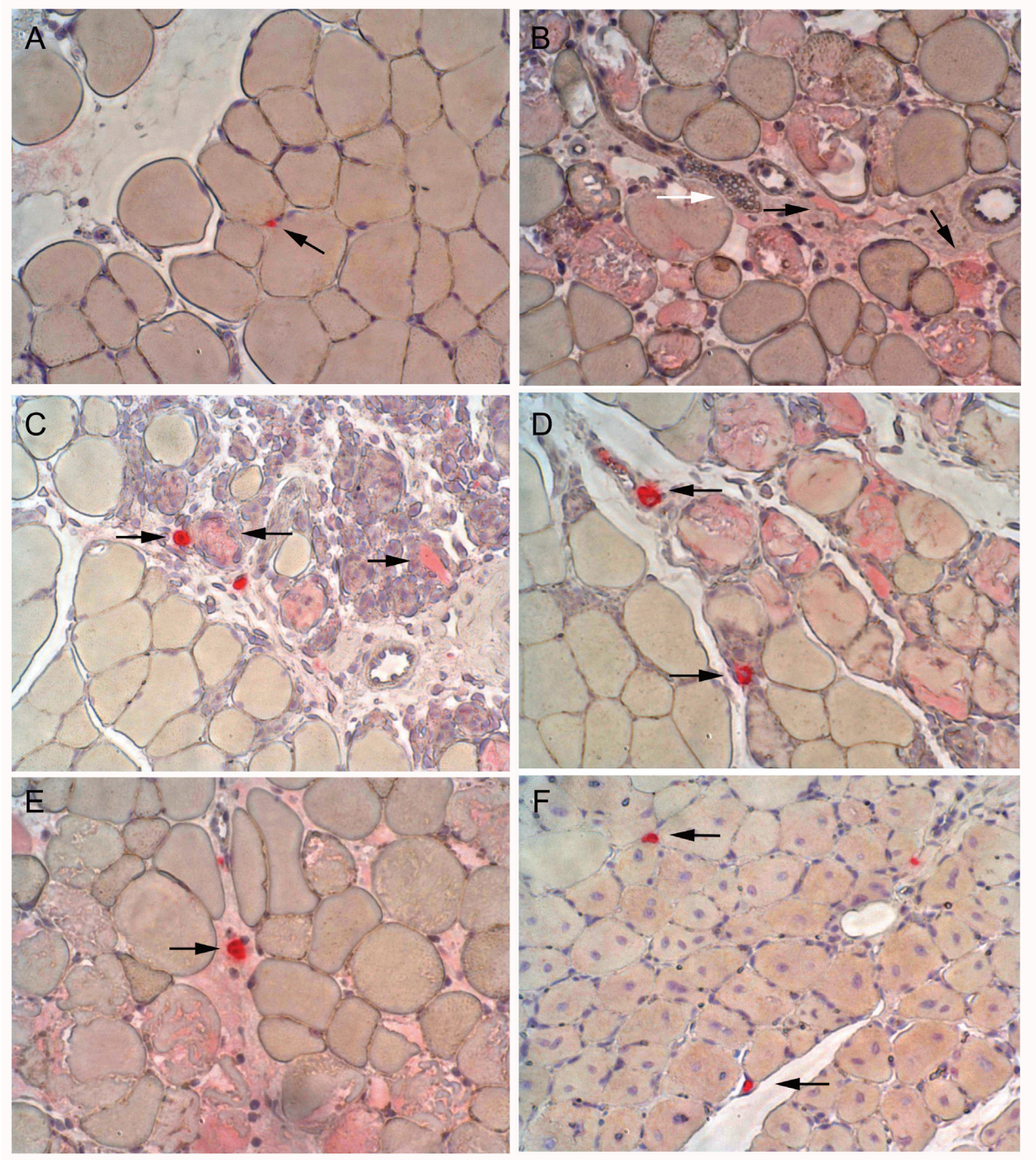

FIGURE 13

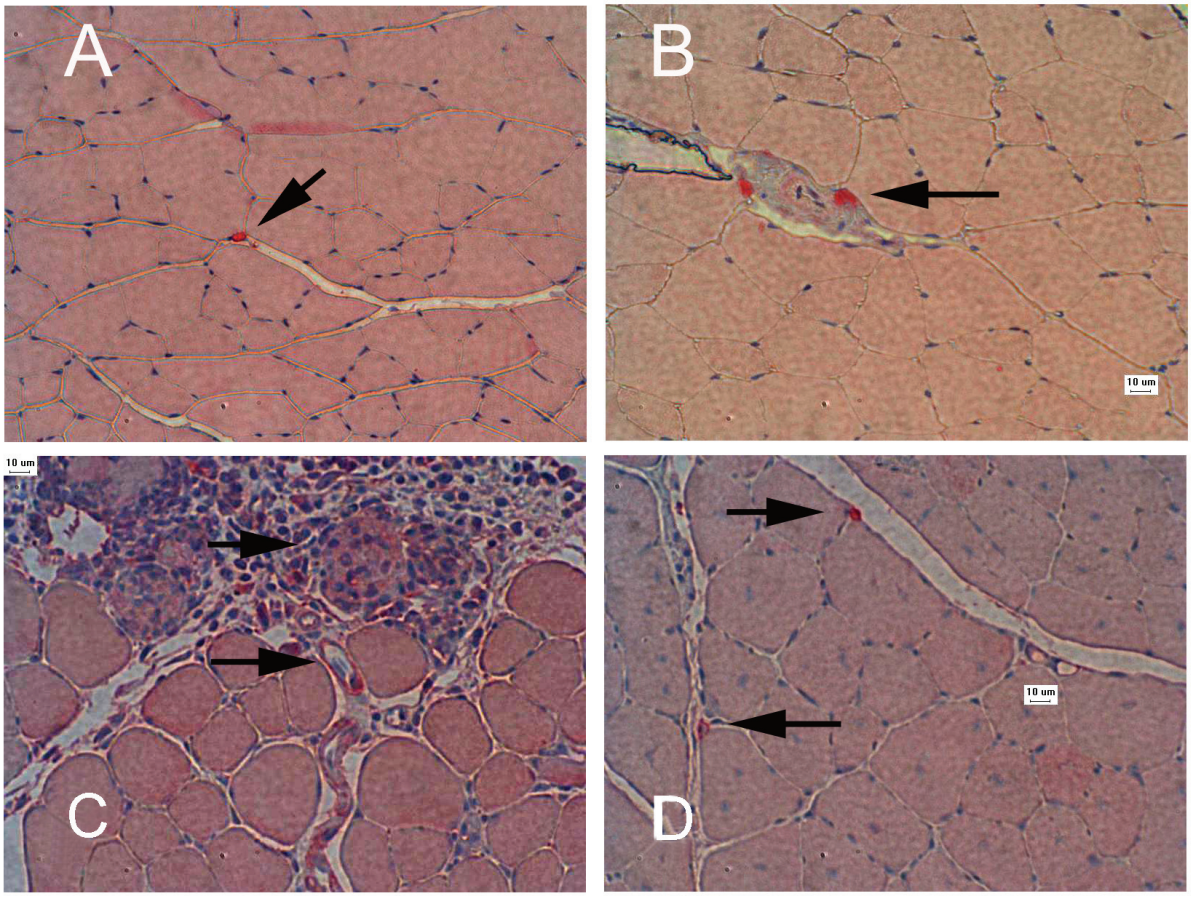

FIGURE 14

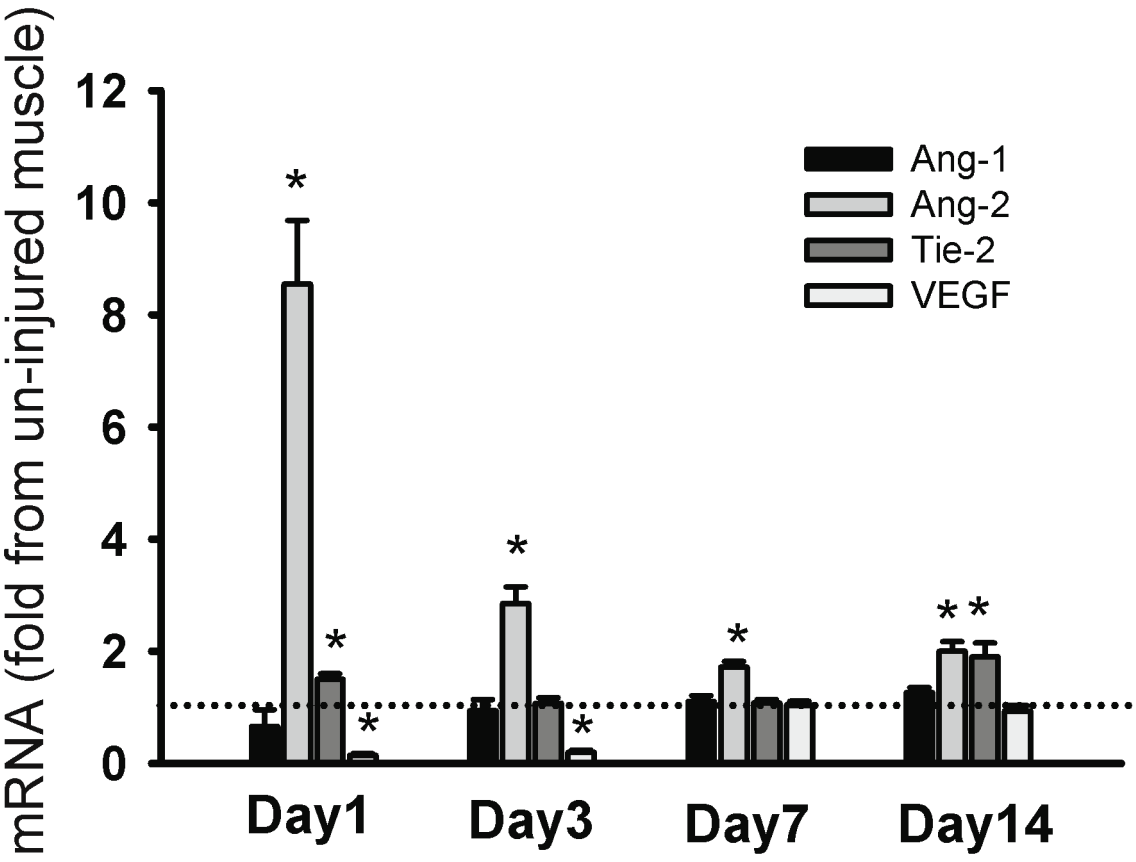
mRNA (fold from un-injured muscle)
12
10
8
6
4
2
0
Ang-1
Ang-2
Tie-2
VEGF
Day1
Day3
Day7
Day14

FIGURE 15

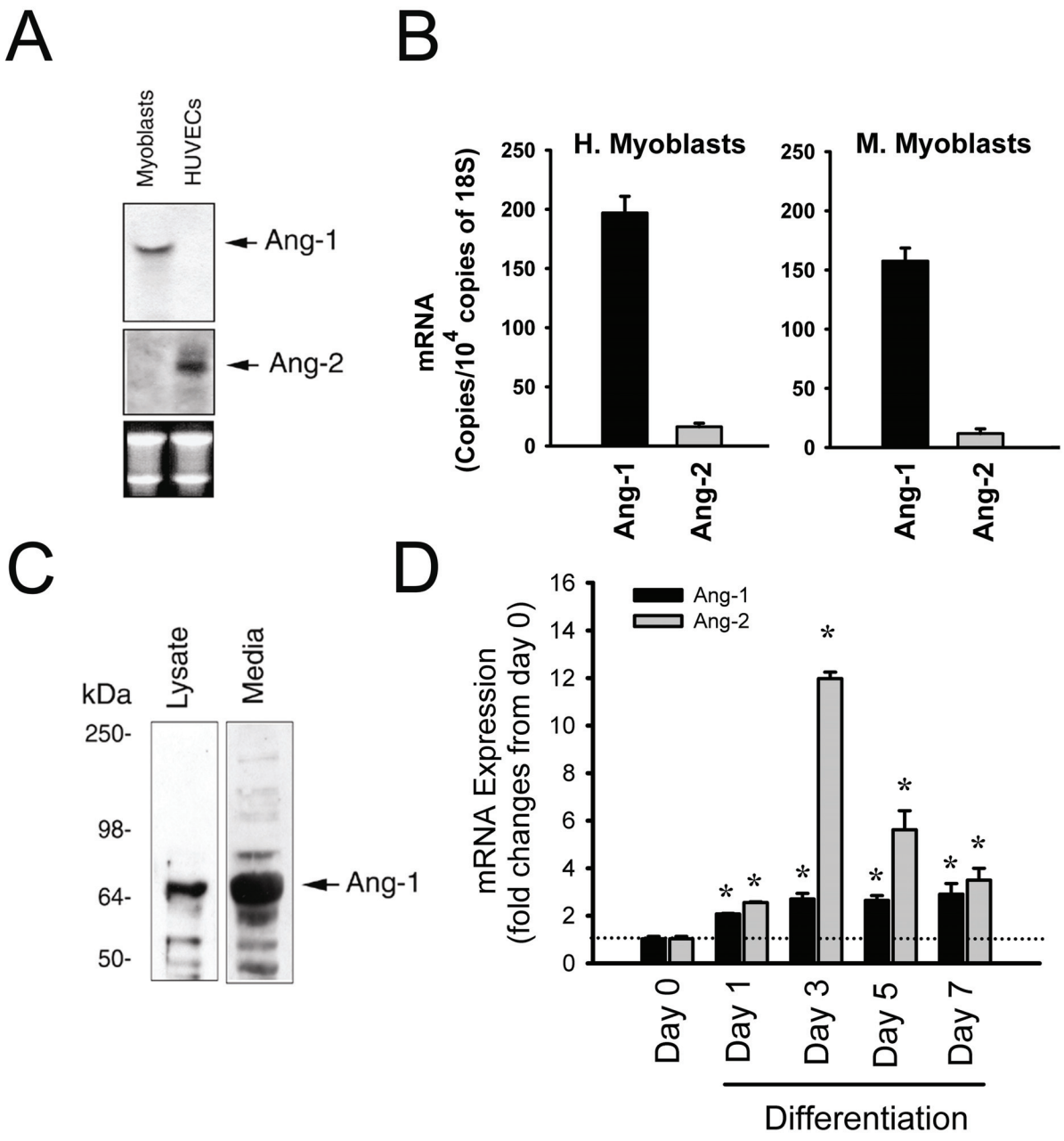
A
Myoblasts
HUVECs
Ang-1
Ang-2
B
H. Myoblasts
M. Myoblasts
mRNA
(Copies/10^4 copies of 18S)
250
200
150
100
50
0
Ang-1
Ang-2
C
kDa
Lysate
Media
250-
98-
64-
50-
Ang-1
D
mRNA Expression
(fold changes from day 0)
Ang-1
Ang-2
16
14
12
10
8
6
4
2
0
*
Day 0
Day 1
Day 3
Day 5
Day 7
Differentiation

FIGURE 16

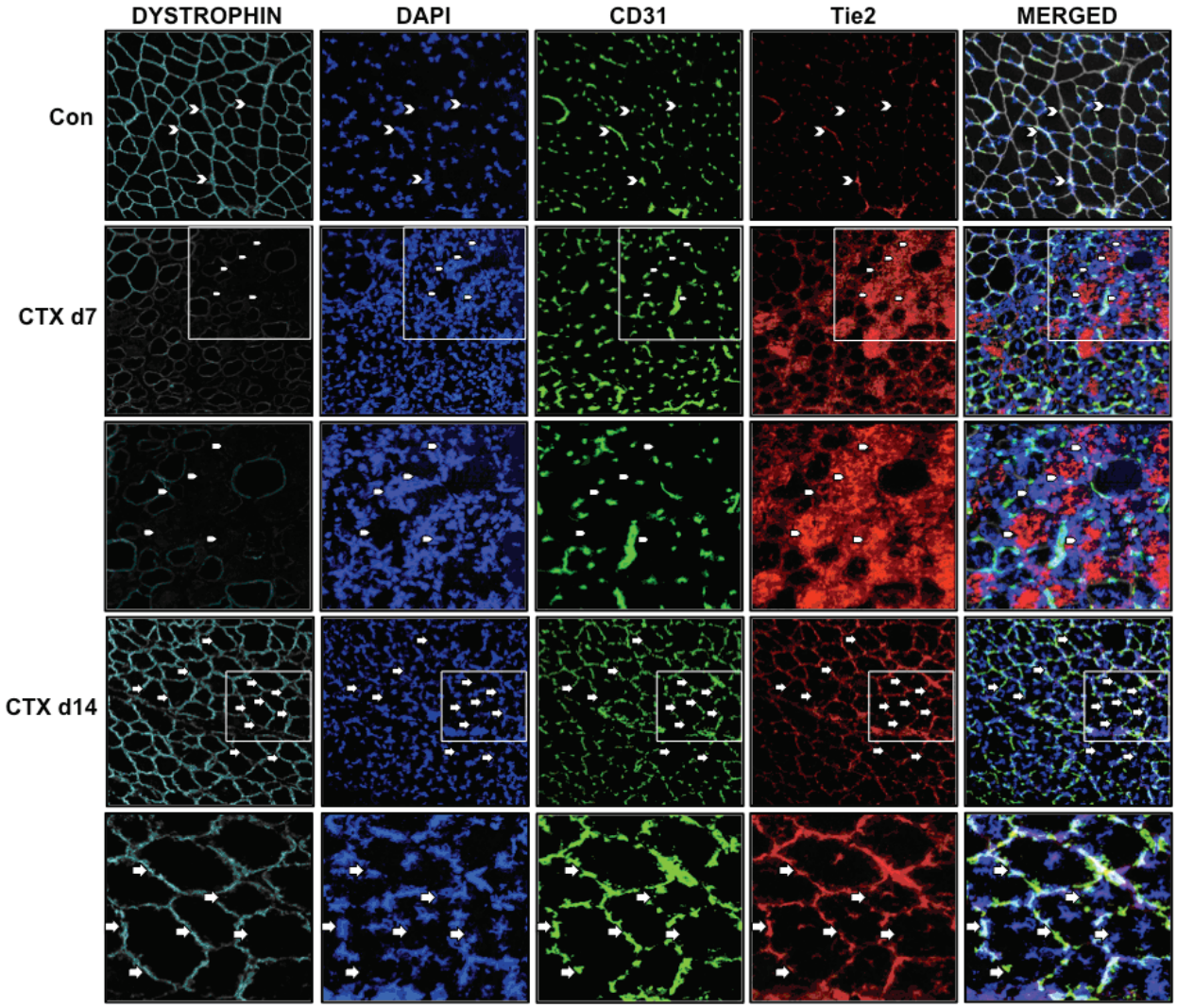

FIGURE 17

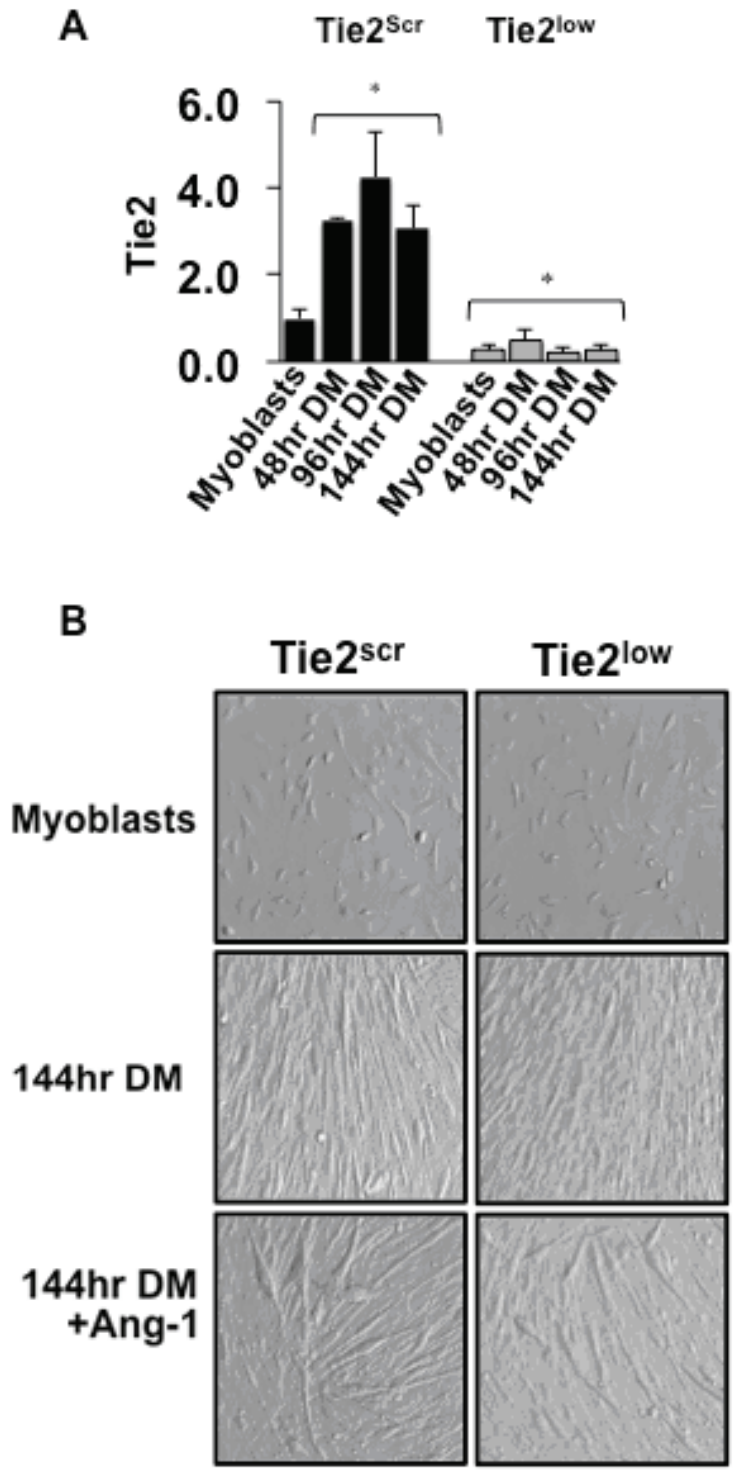
A
Tie2Scr
Tie2low
6.0
4.0
2.0
0.0
Tie2
*
*
Myoblasts
48hr DM
96hr DM
144hr DM
Myoblasts
48hr DM
96hr DM
144hr DM
B
Tie2scr
Tie2low
Myoblasts
144hr DM
144hr DM
+Ang-1

FIGURE 18

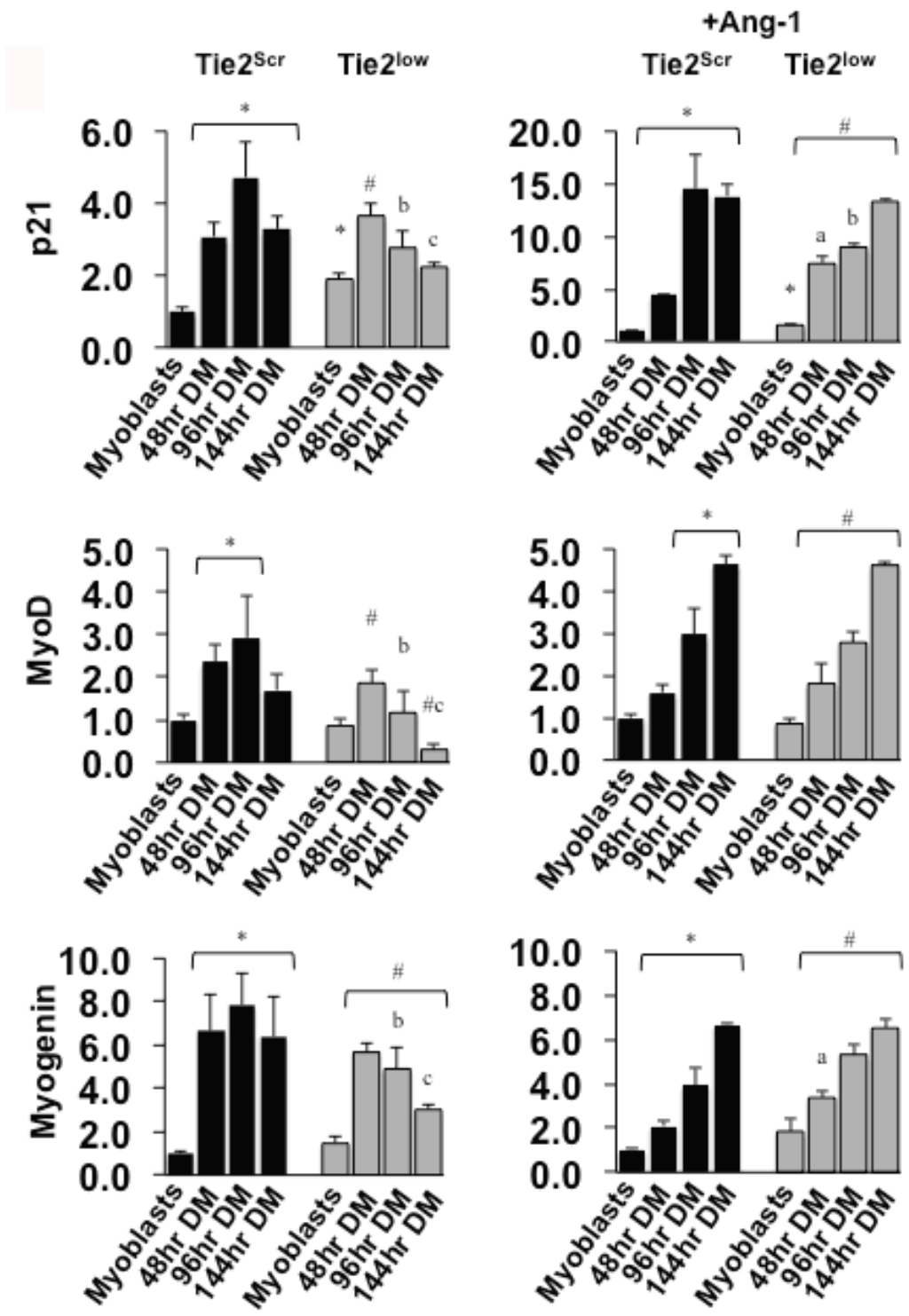
+Ang-1
Tie2Scr
Tie2low
Tie2Scr
Tie2low
p21
MyoD
Myogenin
Myoblasts
48hr DM
96hr DM
144hr DM

FIGURE 19

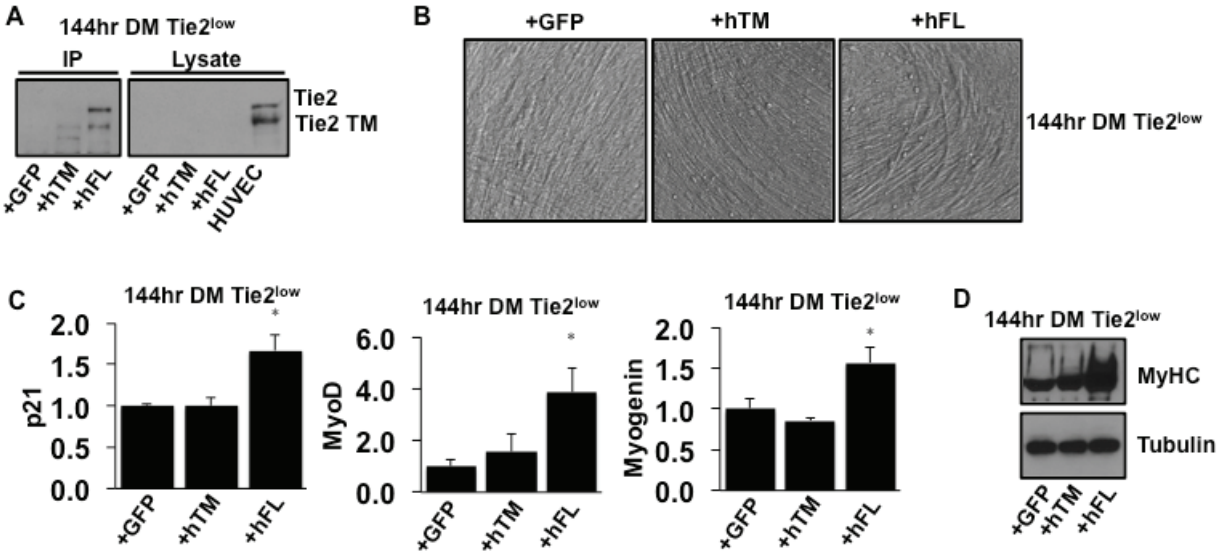
A
144hr DM Tie2low
IP
Lysate
Tie2
Tie2 TM
+GFP
+hTM
+hFL
+GFP
+hTM
+hFL
HUVEC
B
+GFP
+hTM
+hFL
144hr DM Tie2low
C
144hr DM Tie2low
p21
2.0
1.5
1.0
0.5
0.0
+GFP
+hTM
+hFL
144hr DM Tie2low
MyoD
6.0
4.0
2.0
0.0
+GFP
+hTM
+hFL
144hr DM Tie2low
Myogenin
2.0
1.5
1.0
0.5
0.0
+GFP
+hTM
+hFL
D
144hr DM Tie2low
MyHC
Tubulin
+GFP
+hTM
+hFL

FIGURE 20

4.9 FIGURE LEGENDS

Figure 1
Skeletal muscle myoblasts and myotubes express biologically functional Tie2 receptors. Human muscle cells were analyzed via RT-PCR (**A**) and QRT-PCR (**B**) for Tie2 mRNA. *Significantly ($p<0.05$) different from HUVEC. # Significantly ($p<0.05$) different from human or mouse myoblasts. **C**. Tie2 was immunoprecipitated from Ang-1 treated (600ng/ml) human muscle myoblasts and analyzed by western for tyrosine phosphorylation. **D.** Tie-2 phosphorylation on both Tyr^{992} and $Tyr^{1102\text{-}1108}$ by Ang-1 stimulation in human myoblasts is transient.

Figure 2
Regulation of myoblast proliferation by Ang-1
A. Human skeletal myoblasts were exposed to increasing concentrations of Ang-1 or Ang-2 and viable cells were counted 96hours later. *$P<0.05$ compared with control. **B** and **C.** Human skeletal myoblasts were infected with adenoviruses expressing GFP (control), Ang-1 or Ang-2. After 48hours of recovery, equal numbers of cells were plated and viable cells and BrdU absorbance were measured 72h later. *$P<0.05$ compared with Ad-GFP. **D.** Human myoblasts were adenoviral-soluble Tie2 (Ex-Tek) infected and analyzed for cell number and BrdU incorporation in the presence of recombinant Ang-1. *$P<0.05$ compared with control.

Figure 3
Regulation of Tie-2 receptor expression in myoblasts during differentiation
A. BL6 Primary myoblasts were analyzed for Tie2 expression by QRT-PCR at 90% confluence (myoblasts), and with 48-, 96-, and 144-hours of post-confluence differentiation medium exposure (DM). *Significantly ($p<0.05$) different from myoblasts. **B**. Tie2 expression mediated GFP in primary muscle cells isolated from mTmG-Tie2Cre mice at low confluence and 96-hours of post-confluence differentiation medium exposure (96hr DM). **C**. Immunoprecipitation of Tie2 from 90% confluent, fully differentiated (144hr DM), reserve quiescent myoblasts (RC), or control HUVEC cells. **D.** Whole muscle immunofluorescence of transverse sections of plantaris muscle from adult (10-week) mTmG-Tie2Cre mice.

Figure 4
Ang-1 promotes myoblast differentiation
Mouse primary myoblasts were grown to confluence, treated with rhHGF or rhAng-1 in differentiation medium for 24-hours, and analyzed by QRTPCR (**A**) for p21, MyoD, Myogenin, Pgc1-α, and creatine kinase (CK) or by immunoblotting for myosin heavy chain (MHC)(**B**). * Significantly ($p<0.05$) different from myoblast control.

Figure 5
Ang-1 promotes myoblast differentiation

Human muscle cells were infected with adenoviruses expressing GFP or Ang-1. Cells were then grown to confluence and cultured for 7 days in differentiation medium. Cells were analysed for MyoD, myogenin, and MHC mRNA by QRTPCR (**A,B,C**) and proteins by immunoblotting (**D**). *Significantly different ($p<0.05$) from day 0 (myoblasts). # Significantly ($p<0.05$) different from Ad-GFP.

Figure 6
Ang-1 promotes myogenin expression
A myogenin reporter construct (**A**) was co-transfected with empty or MyoD expression plasmids into confluent C2C12 (**B**) and Human (**C**) muscle cells and analyzed for luciferase promoter activity. *Significantly ($p<0.05$) different from empty PGL3-Luc control. # Significantly ($p<0.05$) different from other treatment groups.

Figure 7
Ang-1 stimulates focal adhesion activation in myoblasts. Human skeletal myoblasts were exposed to recombinant Ang-1 and analyzed by western blotting for phosphorylation of paxillin, FAK1, ERK1/2, p38 and SAPK proteins (**A,B**). **C**. Ang-1-treated human myoblasts were analyzed for AKT, mTOR, P70S6 kinase, 4E-BP1 phosphorylation, and phosphorylation of the AP-1 subunit immediate-early response protein c-Jun on Ser^{63} and Ser^{73} (**D**). *Significantly different ($p<0.05$) from 0 min control.

Figure 8
Regulation of myoblast survival and migration by Ang-1
Human skeletal myoblasts were maintained in serum-free medium for 36 hours in the presence of PBS (control) or 600 ng/ml of Ang-1 and cytotoxicity (**A**) and caspase 3/7 activity (**B**) were measured. *$P<0.05$ compared with 15%FBS. # $P<0.05$ compared with PBS. **C.** Measurements of human skeletal myoblasts migration with scratch assay in the presence of PBS (control), recombinant Ang-1 or Ang-2. * $P<0.05$ compared with control. **D.** Skeletal myoblasts were infected with Ad-GFP, Ad-Ang-1 or Ad-Ang-2 and after 48 hours of recovery, confluent cells were wounded with a pipette tip and healing of the wound was measured after 12 hours. * $P<0.05$ compared with Ad-GFP.

Figure 9
Ang-1 overexpression enhances muscle regeneration from injury
Mice received an injection of cardiotoxin and adenoviruses expressing human Ang-1 (Ad-Ang-1, a total of $1.5x10^9$ virus particles/muscle) or adenoviruses expressing GFP (control treatment). The percentage of total fibres injured and recovering (**A**) as well as the percentage of fibres with one, two, or three centralized nuclei (**B**) and myofibre cross sectional area distribution (**C**) were analyzed morphologically 10-days after injury after visualization with H&E.

Figure 10
Ang-1 overexpression enhances muscle force generation after injury

Muscle force production (*in-situ* contractility, N/cm^2) was analyzed 10-days (**A**) and 14-days post injury (**B**).

Figure 11
Ang-1 overexpression enhances muscle regeneration from injury
Immunohistochemistry was performed to identify the number of fibres expressing embryonic myosin heavy chain (eMHC) and Von Willibrand Factor (**A**). Fibres expressing eMHC were quantified as a percentage of the total fibre number (**B**). # Significantly different ($p<0.05$) from Adenoviral GFP treatment. * Significantly ($p<0.05$) different from Ad-GFP Control. # Significantly ($p<0.05$) different from day matched Ad-GFP control.

Figure 12
Ang-1 overexpression stimulates capillary formation in regenerating muscle
Real time PCR quantification of two markers of endothelial cells (Tie-1 and PECAM1) measured 10 days after injury and treatment with Ad-GFP and Ad-Ang-1 viruses. Results are expressed as fold change from those measured in un-injured muscle. *$P<0.05$ compared with un-injured muscle. # compared with Ad-GFP.

Figure 13
Ang-1 is produced by muscle myoblasts and differentiating myotubes. Un-injured TA (**A**) and TA muscles obtained after 1 day (**B, C**), 3 days (**D**), 7 days (**E**) and 14 days (**F**) of cardiotoxin-injected were immunostained for Ang-1 using a selective antibody. Black arrows are pointing to positive (pink) staining.

Figure 14
Ang-2 is produced by muscle myoblasts and differentiating myotubes. Un-injured TA (**A, B**) and TA muscles obtained after 1 day (**C**), and 14 days (**D**) of cardiotoxin-injected were immunostained for Ang-2 using a selective antibody. Black arrows are pointing to positive (pink) staining.

Figure 15
Changes in angiopoietin, Tie-2 receptor and VEGF mRNA levels during injury and regeneration of TA muscle
QRT-PCR analysis of Ang-1, Ang-2, Tie2, and VEGF mRNA levels of TA during the time course of cardiotoxin-induced injury and regeneration. Data are expressed as fold changes from un-injured. * Significantly ($p<0.05$) different from un-injured muscle.

Figure 16
Ang-1 and Ang-2 production by skeletal myoblasts
Northern blot (**A**) and QRT-PCR (**B**) analysis of Ang-1 and Ang-2 mRNA in human skeletal myoblasts. **C**. Human myoblast lysates and conditioned medium were analyzed for Ang-1 by immunoblotting. **D**. Differentiating human muscle cells were analyzed via QRT-PCR for Ang-1 and Ang-2 mRNA. * Significantly ($p<0.05$) different from day 0 Controls.

Figure 17

Regenerating myofibre centralized nuclei express Tie2. Tibialis Anterior muscle regenerating for 14-days after cardiotoxin was immunofluorescence labeled for dystrophin, nuclei (DAPI), capillaries (CD31), and Tie2 and visualized by confocal microscopy. White Chevron in Con indicates predominant Tie2-CD31 co-staining at baseline. Immunofluorescence of regenerating muscle revealed Tie2 staining in capillaries, mononuclear infiltrating cells, and centralized nuclei in the initial week of regeneration (CTX d7; 200X, White Box 400X-subsequent row). IF of d14 regenerating muscle for Tie2 staining independent of capillary staining in centralized nuclei (White arrows; 200X, White Box 400X-subsequent row).

Figure 18

Tie2 is required for muscle cell differentiation in the absence of exogenous Ang-1

Stable Tie2 knockdown ($Tie2^{low}$) or control/scrambled ($Tie2^{Scr}$) primary muscle myotubes were generated by retroviral infection and antibiotic selection (**A**). **B**. Stable myoblasts were grown to confluence and treated with recombinant Ang-1 or PBS control daily for 144-hours of differentiation.

Figure 19

Tie2 is required for muscle cell differentiation in the absence of exogenous Ang-1.

Cells were analyzed by QRT-PCR at pre-treatment 90% confluence and after 48-, 96-, and 144-hours of post-confluent differentiation for p21, MyoD, and myogenin. * Significantly ($p<0.05$) different from $Tie\text{-}2^{Scr}$ 90% confluent myoblast control. # Significantly ($p<0.05$) different from $Tie\text{-}2^{low}$ 90% confluent myoblast control. ^{a,b,c} Significantly ($p<0.05$) different from differentiation-hour (48-, 96-, 144-hour, respectively) matched $Tie\text{-}2^{Scr}$.

Figure 20

Tie2 kinase activity is a time-dependent requirement for muscle cell differentiation *in vitro*. Stable Tie2 knockdown ($Tie2^{low}$) myoblasts were grown to confluence and allowed to differentiate for 24-hours before serial transfections (24- and 48-hours DM) with either GFP, human Tie2 mutant truncated after the transmembrane domain and lacking the entire cytosolic domain (+hTM), or full-length human Tie2 (+hFL). Transfected cells were then allowed to continue differentiating for the full 144 hours time-course. Immunoprecipitation followed immunoblotting were used to verify Tie-2 receptor expression (A). Microscopy was used to visualize the differentiation of myoblasts (**B**). Cells were analyzed by QRT-PCR after 144-hours of post-confluent differentiation for p21, MyoD, and Myogenin (**C**) and by immunoblotting for MyHC (**D**). * Significantly ($p<0.05$) different from GFP control.

4.10 TABLE 1: Primers used for real-time PCR measurements of Myogenin, Pax7, Creatine Kinase, PECAM1, Tie-1 and β-Actin expressions in murine tibialis anterior muscle samples.

Gene	Accession #
Myogenin	NM_031189
Forward 5'-GCACTGGAGTTCGGTCCCAA-3'	
Reverse 5'- TATCCTCCACCGTGATGCTG-3'	
Pax7	NM_011039
Forward 5′-TCCATCAAGCCAGGAGACA-3′	
Reverse 5′-AGGAAGAAGTCCCAGCACAG-3′	
Creatine Kinase	NM_007710
Forward 5'- ACGAGAACCTCAAGGGTGGA-3	
Reverse 5'- CACGGACAGCTTCTCCACTG-3'	
PECAM1	NM_001032378
Forward 5'-CCCAAGGTGACACTGGACAA3'	
Reverse 5'-ATGGGGAATTCCATGAGCAC-3'	
Tie-1	NM_001032378
Forward 5'- CCCAAGGTGACACTGGACAA-3'	
Reverse 5'- ATGGGGAATTCCATGAGCAC-3'	
PECAM1	NM_011587
Forward 5'- CAGCATGAAACTTCGCAAGC-3'	
Reverse 5'- AGAAGTCGATACGCGGCATT-3'	
β-Actin	NM_007393
Forward 5'- AACCGTGAAAAGATGACCCAG-3'	
Reverse 5'- CACAGCCTGGATGGCTACGTA-3'	

4.11 TABLE 2: Genes whose expression is regulated by Ang-1 in skeletal myoblasts.

Accession	Symbol	Definition	Ad-Ang-1/ Ad-GFP	p value
Genes which are induced by Ang-1 in skeletal myoblasts				
NM016111	TEL2	Telomere maintenance 2 homology	16.1	0.0000
NM001024629	NRP1	Neuropilin1 transcript variant 3	12.3	0.0000
NM201414	APP	Amyloid beta A4 precursor	7.3	0.0000
NM002007	FGF4	Fibroblast growth factor 4	3.9	0.0000
NM032645	RAPSN	Receptor-associated protein of synapse	3.4	0.0000
NM014067	LRP16	Leukemia related protein 16	3.4	0.0002
NM032645	C10orf33	Chromosome 10 open reading frame 33	3.2	0.0000
NM032340	C6orf125	Chromosome 6 open reading frame 125	3.1	0.0000
NM199295	APITD1	Apoptosis-induced TAF9-like domain 1	3.0	0.0001
NM181877	ZSCAN2	Zinc finger and SCAN domain containing	3.0	0.0000
NM000230	LEP	Leptin	2.9	0.0000
NM001145	ANG	Angiogenin	2.8	0.0000
NM182480	COQ6	Coenzyme Q6 homolog	2.8	0.0002
NM016401	C11orf73	Chromosome 11 open reading frame 73	2.6	0.0000
NM001809	CENPA	Centromere protein A, 17 kDa	2.6	0.0003
NM002982	CCL2	Chemokine (C-C motif) ligand 2	2.6	0.0001
NM002587	PCDH1	Protocadherin 1	2.6	0.0045
NM014935	PEPP3	Phosphoinositol 3-phosphate binding protein	2.6	0.0058
NM001013436	MPST	Mercaptopyruvate sulfurtransferase	2.5	0.0007
NM182687	PKMYT1	Protein kinase, membrane associated	2.5	0.0094
NM003178	SYN2	Synapsin II	2.5	0.0134
NM031300	MXD3	MAX dimerization protein 3	2.5	0.0000
Genes which are downregulated by Ang-1 in skeletal myoblasts				
NM002155	HSPA6	Heat shock 70 kDa protein 6	0.02	0.0000
NM178539	TAFA2	Chemokine-like protein TAFA-2	0.06	0.0000
NM013453	SPANXA1	Spanx family member A1	0.08	0.0000
NM022661	SPANXC	Spanx family member C	0.10	0.0000
NM145665	SPANXE	Spanx family, member E	0.12	0.0000
NM032461	SPANXB1	Spanx family, member B1	0.14	0.0000
NM002575	SERPINB2	Serine peptidase inhibitor, clade B	0.14	0.0000
NM000488	SERPINC1	Serine peptidase inhibitor, clade C	0.14	0.0000
NM016084	RASD1	RAS, dexamethasone-induced 1	0.15	0.0000
NM005345	HSPA1A	Heat shock 70kDa protein 1A	0.15	0.0000
NM005252	FOS	V-fos FBJ murine osteosarcome viral oncogene homolog	0.15	0.0000
NM005346	HSPA1B	Heat shock 70kDa protein 1B	0.18	0.0000
NM006072	CCL26	Chemokine ligand 26	0.19	0.0002
NM000582	SPP1	Secreted phosphoprotein 1	0.20	0.0000
NM003545	HIST1H4E	Histone 1, H4e	0.23	0.0000
NM153292	NOS2A	Nitric oxide synthase 2A	0.24	0.0000
NM018602	DNAJA4	DNAJ (Hsp40) homolog	0.25	0.0000

NM002923	RGS2	Regulator of G-protein signaling 2	0.25	0.0000
NM005980	S100P	S100 calcium binding protein P	0.25	0.0001
NM000506	F2	Thrombin	0.25	0.0001
NM139314	ANGPTL4	Angiopoietin-like 4	0.26	0.0001
NM194293	CMYA1	Cardiomyopathy associated 1	0.27	0.0002
NM138340	ABHD3	Abhydrolase domain containing 3	0.28	0.0001
NM130386	COLEC12	Collectin subfamily member 12	0.29	0.0001
NM152782	SUNC1	Sad1 and unc84 domain containing 1	0.29	0.0001
NM018324	THEDC1	Thioesterase domain containing 1	0.29	0.0000
NM003518	HIST1H2BG	Histone 1, H2bg	0.29	0.0000
NM176870	MT1M	Metallothionein 1M	0.30	0.0001
NM000601	HGF	Hepatocyte growth factor	0.30	0.0001

CHAPTER 5

ORIGINAL CONTRIBUTIONS TO SCIENTIFIC KNOWLEDGE AND DISCUSSION

5.1 Regulation of angiopoietin expression by bacterial lipopolysaccharide

Contradictory results have been reported with respect to the influence of pro-inflammatory cytokine on Ang-1 expression in various cells (36, 170). In chapter 2, we describe for the first time the *in-vivo* alterations in Ang-1 expression in three different organs (liver, lung and diaphragm) following the systemic administration of *E. coli* LPS (animal model of septic shock). Our results (chapter 2, figures 1 and 2) indicate that LPS administration elicits within 12 hours a sustained and substantial decline in Ang-1 expression (both at mRNA and protein levels) in these three organs. Interestingly, LPS administration also triggered significant decline in Tie-2 receptor expression and phosphorylation in the liver, lung and the diaphragm (chapter 2, figure 3). These results suggest that the Ang-1/Tie-2 receptor pathway is functionally inhibited in septic shock in part due to downregulation of the ligand (Ang-1) and the receptors (Tie-2). The implications of downregulation of tissue Ang-1 and Tie-2 expression by LPS administration to the pathogenesis of vascular collapse in septic shock remain speculative and further studies are needed to explore this issue. We propose based on the facts that Ang-1 has potent anti-inflammatory effects and strong inhibitory influences on vascular leakage that down-regulation of Ang-1 and Tie-2 in septic shock contributes to the augmentation of cytokine cascade, tissue injury and increased vascular leakage observed in patients with septic shock. Our findings also suggest that restoration of Ang-1 and/or Tie-2 expression may reverse several hemodynamic manifestations of septic shock. Witzenbichler *et al.* (373) tested this possibility by pre-treating mice with intravenous injection of adenoviruses expressing Ang-1 (Ad-Ang-1). Adenoviruses expressing GFP (Ad-GFP) were used as control.

Two days after the administration of these viruses, animals received i.p. injection of LPS. These authors reported that LPS-induced mortality, acute lung injury, adhesion molecule expression as well as hypotension were all significantly attenuated in animals who received Ad-Ang-1 compared with Ad-GFP. These results clearly illustrate the utility of enhancing Ang-1 expression as a potent therapeutic tool in the treatment of septic shock.

The exact mechanisms through which LPS administration alters *in-vivo* expression of Ang-1 and Tie-2 receptors was not evaluated in our study, however, we were able to identify the roles of NFκB transcription factor in LPS-induced alterations in Ang-1 expression by using cultured pulmonary epithelial and skeletal muscle cells. The rationale behind focusing on this transcription factor stems from the fact that it is central participant in modulating the expression of many of the immunoregulatory mediators involved in the development of organ failure in septic patients (6). Inhibition of NFκB transcription factor using adenoviruses expressing dominant negative form of IKKα had no effect on the response of Ang-1 expression to LPS exposure. These results are in accordance with the results of Brown *et al.* (36) who reported the absence of NFκB binding sites on human Ang-1 promoter but identified ESE-1 transcriptional factor as an important inducer of Ang-1 expression in fibroblasts exposed to TNFα, IL1β or LPS. These authors also suggested that NFκB may indirectly induce Ang-1 expression in fibroblasts through the ESE-1 transcription factor. We observed (chapter 2, figure 6) significant induction of ESE-1 expression in epithelial and skeletal muscles exposed to LPS and that inhibition of NFκB eliminated this response. These results confirm that ESE-1 is substantially

induced by LPS exposure and that this response is mediated in part through NFκB. These results also rule out an important role for ESE-1 transcription factor for LPS-induced alterations in Ang-1 expression in epithelial and skeletal muscle cells.

It should be emphasized that although *in-vitro* and *in-vivo* exposures to LPS elicited qualitatively similar decline in Ang-1 expression in cultured skeletal muscle cells and *in-vivo* Ang-1 expression, LPS exposure significantly induced Ang-1 expression in cultured pulmonary epithelial cells. We have no clear explanation for the difference between the i*n-vitro* and *in-vivo* LPS-induced changes in epithelial Ang-1 expression but we speculate that in cultured epithelial cells, primary signaling events triggered by activation of Toll-like 4 (TLR4) receptors may have been involved in the induction of Ang-1 expression whereas secondary mediators released in response to LPS injection such as TNFα may be responsible for inhibition of Ang-1 expression in the lungs of LPS-injected mice. This is based on the observations in osteoclasts where TNFα significantly down-regulates Ang-1 expression (171).

Several studies have pointed out that pro-inflammatory mediators such as TNFα and VEGF induce endothelial cell Ang-2 expression (130, 180, 220). Few studies have addressed the *in-vivo* regulation of Ang-2 expression in relation to pro-inflammatory mediators. Karmpaliotis *et al*. (170) described no significant alterations in pulmonary Ang-2 expression in mice receiving nebulized LPS suggesting that in their model of acute lung injury, pulmonary Ang-2 production is not elevated. However, Orfanos and colleagues (254) found a significant elevation of plasma Ang-2 levels in patients with septic shock and that plasma Ang-2 levels correlate positively with plasma TNFα and IL6. When these authors exposed human

pulmonary endothelial cells to TNFα, IL6 and LPS, they found significant decline in Ang-2 expression. Orfanos *et al.* (254) concluded that Ang-2 production is elevated in patients with sepsis, however, pulmonary vascular endothelial cells is not involved in this response. Elevated plasma Ang-2 levels in patients with sepsis have been confirmed by others (68, 263). To investigate organ-specific regulation of Ang-2 production in septic shock and to assess the involvement of NFkB transcription factor in this production, we studied Ang-2 expression in mice injected with LPS and in cultured pulmonary epithelial and skeletal muscle cells exposed to LPS. My results indicate that Ang-2 production is elevated in three organs (lungs, liver and diaphragm) in mice injected with LPS and in cells exposed to LPS (chapter 2, figures 1, 4 and 5). Furthermore, our results indicate that inhibition of NFκB transcription factor in cultured pulmonary epithelial and skeletal muscle cells resulted in complete inhibition of LPS-induced Ang-2 expression in these cells thereby confirming that the NFκB transcription factor is critical to the regulation of Ang-2 expression by LPS. This finding is interesting because analysis of human Ang-2 promoter revealed abundant putative binding sites for several transcription factors including Ets and GATA factors, AP1 and Smad family of factors but not NFκB transcription factor (140). Hasegawa *et al.* (137) have confirmed the importance of Ets-1 transcription factor in regulating Ang-2 promoter activity in response to VEGF stimulation. On the basis of these results, I propose that NFkB transcription factor regulates Ang-2 promoter activity indirectly and most probably through interactions with the Ets transcription factor family. Such interactions where Ets factors promote NFκB-driven

transcription of genes previously not known to be responsive to NFκB have recently been described by De Sieveri *et al.* in Jurkat cells (74).

There are several functional implications to the induction of Ang-2 production in septic animals. First, enhanced Ang-2 levels will result in even greater functional inhibition of the Ang-1/Tie-2 receptor pathway than that caused by reduction of Ang-1 and Tie-2 expression in septic animals because of the antagonistic effect of Ang-2 on Ang-1-induced Tie-2 phosphorylation in endothelial cells. Second, although there is increasing evidence that circulating levels of Ang-2 are elevated in patients with septic shock or severe sepsis (68, 254, 263), the cellular origin of Ang-2 in these studies remains unclear. We report in chapter 2 that Ang-2 mRNA and protein levels rose significantly at least in three organs (liver, lung and diaphragm) in response to LPS injection in mice suggesting that elevated Ang-2 production in patients with septic shock or severe sepsis is derived from several organs and is not limited only to the vasculature. Third, endothelial cells store Ang-2 in specialized endothelial storage granules (Weibel–Palade bodies) in which it co-localizes with von Willebrand Factor (vWF)(98). Certain mediators such as thrombin and vasopressin are able to liberate Weibel–Palade body stores very rapidly thereby making Ang-2 available for rapid responses including changes in permeability, coagulation or inflammation. The contribution of this rapid release pathway to elevated circulating levels of Ang-2 in patients with septic shock remains unknown, however, my study points out that Ang-2 mRNA levels rose significantly within 6hrs of LPS administration suggesting that transcriptional regulation of Ang-2 production in septic shock is quite rapid and that

transcriptional activation of Ang-2 works in conjunction with Weibel-Palade body stores of Ang-2 to quickly increase local or systemic levels of this protein.

Ang-3 and Ang-4 are interspecies orthologs and represent the third and fourth members of the angiopoietin family. Initial studies showed that Ang-4 phosphorylates Tie-2 receptors whereas Ang-3 inhibits this phosphorylation (359). It was concluded on the basis of these results that Ang-4 is a Tie-2 agonist whereas Ang-3 was proposed as a Tie-2 antagonist (359). However, a more recent study by Lee *et al.* (202) concluded that both Ang-3 and Ang-4 are Tie-2 agonists and both produce strong angiogenesis responses. Little is known about the regulation of Ang-3 and Ang-4 expression. Ang-4 expression increases in response to growth factors and hypoxia (112, 215). Hypoxia was shown to induce Ang-3 expression in lung, liver, cerebellum and heart of rats where Ang-1 expression is significantly declined (4). Our study is the first to address the influence of LPS on Ang-3 and Ang-4 expression in mice and in cultured epithelial and muscle cells, respectively. Chapter 2 illustrates that LPS exposure both *in-vivo* and *in-vitro* elicits significant decline in Ang-3 and Ang-4 expressions in a fashion similar to that observed with Ang-1. Moreover, we found that LPS-induced decline in Ang-4 in cultured epithelial and skeletal muscle cells is not mediated by NFκB transcription factor since inhibition of this factor had no influence on the response to LPS exposure. The similarity in the decline of Ang-1, Ang-3 and Ang-4 expressions by LPS raises the possibility that Ang-3 and Ang-4 may play a protective effect in promoting endothelial survival and attenuating inflammatory responses in a fashion similar to that elicited by Ang-1. It is also possible that attenuation of Ang-3 and Ang-4 expressions by LPS may further

contribute to the functional inhibition of Tie-2 receptor activation in sepsis and may contribute to the pathogenesis of vascular dysfunction and leakage in septic shock. This argument is supported in part by the recent observations that Ang-3 and Ang-4 stimulate the pro-survival PI-3 kinase/AKT pathway in cultured endothelial cells (202). However, further studies are needed to assess the influences of Ang-3 and Ang-4 on the inflammatory responses of endothelial cells and to confirm whether these angiopoietins play a protective role in preventing vascular leakage and attenuation of leukocyte adhesion to endothelial cells.

5.2 Angiogenic factors in limb muscles of chronic obstructive pulmonary disease patients: roles of angiopoietin-2

Despite the importance of angiogenesis in determining capillary density of skeletal muscles and consequently the relationship between oxygen delivery and impaired skeletal muscle performance in COPD patients, no comprehensive studies have so far been published describing the status of angiogenesis vs. angiostatic factors in limb muscles of these patients. Only VEGF, due to its critical importance in promoting angiogenesis, has received a moderate attention. The results in this regard are contradictory since one study described a significant inhibition of VEGF expression while another study has documented no difference in VEGF expression among limb muscles of COPD patients and control subjects (25, 157). In chapter 3, we investigated VEGF as well as Ang-1 and Ang-2 expressions in *vastus lateralis* muscle biopsies from patients with moderate COPD and control subjects. Our results reveal that while VEGF and Ang-1 expressions are not different, that of Ang-2 is significantly higher in muscles of COPD patients compared with control subjects.

Elevated Ang-2 expression correlates with the severity of COPD and with muscle TNFα levels. Initially, our interpretation of these results was that increased Ang-2 production in limb muscles of COPD patients was a result of local and systemic inflammation, however, *in-vitro* experiments in cultured skeletal myoblasts revealed that TNFα was not able to induce Ang-2 expression in these cells, whereas hydrogen peroxide triggered significant induction of Ang-2 mRNA levels and Ang-2 protein secretion in the media of these cells. I concluded on the basis of these results that oxidative stress is likely to be an important factor in inducing Ang-2 expression in skeletal muscles of COPD patients.

Chapter 3 reveals several novel aspects of angiopoietin biology in skeletal muscles.

First, it shows for the first time that human and murine skeletal muscle progenitor cells produce Ang-2 under normal conditions. Dallabrida *et al*. (65) were unable to detect Ang-2 mRNA levels in primary skeletal myoblasts while my results clearly indicate that Ang-2 is produced by human skeletal myoblasts and that Ang-2 protein can be detected in the culture media of these cells using selective ELISA. Interestingly, we found that Ang-2 production increases significantly during differentiation of skeletal myoblasts into myotubes suggesting that mature muscle fibres produce even greater levels of Ang-2 than myoblasts. A general implication of this finding is that Ang-2 production is not restricted to endothelial cells and that skeletal muscles represent another source of Ang-2 that may contribute to circulating levels of Ang-2.

Second, chapter 3 also indicates that Ang-2 promotes skeletal myoblast survival by selectively inhibiting apoptosis and that this effect is mediated through activation of the PI-3 kinase/AKT and the ERK1/2 MAPK pathways. Whether this anti-apoptotic effect of Ang-2 is triggered through Tie-2 receptors and/or integrin ligation was not investigated in chapter 2. Both, Tie-2 receptors and integrins are capable of inducing phosphorylation of AKT and ERK1/2 pathways and both can promote cellular survival. The importance of Tie-2 receptors in angiopoietin signaling in skeletal muscle progenitors is fully explored in chapter 4. The observation that Ang-2 promotes skeletal myoblasts survival implies that enhanced Ang-2 production in skeletal muscles of COPD patients is a positive adaptive response designed to maintain the population of muscle progenitor cells and to improve regenerative capacity of skeletal muscles of these patients.

Third, we show for the first time that Ang-2 significantly enhances differentiation of skeletal muscle progenitor cells. Indeed, over-expression of Ang-2 in skeletal myoblasts triggered significant induction of MyoD and myogenin expressions throughout the differentiation process and the substantial induction of muscle-specific genes (creatine kinase and myosin heavy chain) during the late phase of differentiation. The mechanism through which Ang-2 induces differentiation was not fully investigated. In chapter 4, we will demonstrate that Ang-1, like Ang-2, promotes myoblast differentiation to myotubes. One possible mechanism through which Ang-2 promotes myoblast differentiation is through upregulation of MyoD transcription. This is likely the mechanism since we have observed significant upregulation of MyoD expression by Ang-2 in the myoblast phase prior to the initiation of the

differentiation program. Another possibility is that Ang-2 acts indirectly to induce the expression of secondary mediators and that those mediators induce the differentiation program in myoblasts. One such mediator is VEGF receptor neuropilin 1 (NRP1) whose expression is significantly induced by Ang-2 in skeletal myoblasts. NRP1 receptors are expressed on skeletal muscle progenitors and they are likely to mediate positive interactions between the VEGF and Ang-2 pathways resulting in augmentation of the differentiation program in skeletal progenitor cells. This possibility should be tested in future studies by measuring the influence of Ang-2 on myoblast differentiation in the presence and absence of NRP1 neutralizing antibody or using myoblasts in which NRP1 expression is downregulated by using siRNA oligos.

5.3 Ang-1 promotes muscle regeneration and myoblast differentiation through complementary pathways involving Tie-2

Despite the fact that chapter 3 reveals important and novel effects of Ang-2 on skeletal myoblast differentiation and survival, many questions regarding angiopoietins and skeletal muscle regeneration remained un-answered. The first and logical question is whether Ang-1, like Ang-2, promotes skeletal myoblast differentiation and whether this effect is mediated through Tie-2 receptors. Furthermore, in chapter 3 the existence of Tie-2 receptors in skeletal muscle cells and the involvement of these receptors in the biological effects of Ang-2 on skeletal myoblasts were not addressed. Chapter 4 uncovered two important concepts

regarding the functional importance of the Ang-1/Tie-2 axis in skeletal muscle regeneration.

First, our study is the first to document the existence and the functional significance of Tie-2 receptors in skeletal muscle myogenesis program. We identified the full-length Tie-2 receptor transcripts and detected Tie-2 protein in human and murine skeletal myoblasts and we found that Tie-2 receptor expression is significantly induced during myoblast differentiation into myotubes. More intriguing is our finding that Tie-2 receptors are expressed on the surface of mature skeletal muscle fibres. To document the existence of these receptors in mature skeletal muscle fibres in *in-vivo* settings, we conducted experiments in collaboration with Dr. Kontos's group at Duke University who developed special transgenic mice possessing a double-fluorescent Cre reporter consisting of membrane-targeted tandem dimer Tomato (mT) expressed at baseline and membrane-targeted green fluorescent protein (mG) expressed following Cre-mediated excision. These mice were cross bred with homozygous Tie2-Cre transgenic mice to produce transgenic mice in which activation of the Tie-2 promoter will result in Cre-mediated excision of the mTomato gene and the induction of mGFP expression thereby allowing the visualization of Tie-2 promoter activity at specific developmental time points. Using these mice, we were able to show heterogeneous GFP expression in mature skeletal muscles indicating scattered Tie-2 expression at some point during the life of these muscle fibres.

Despite the novelty of our findings regarding Tie-2 receptor expression in skeletal myoblasts and mature skeletal muscle fibres, it was essential for us to address whether muscle-specific Tie-2 receptors are responsive to Ang-1 by undergoing

selective autophosphorylation of tyrosine residues. We were able to answer this question by exposing cultured skeletal myoblasts to exogenous Ang-1 and observing a significant increase in Tie-2 receptor phosphorylation of specific tyrosine residues including 1102-1108 and 992. These same tyrosine residues are activated in human endothelial cells exposed to Ang-1 indicating that muscle-specific Tie-2 receptors are activated by Ang-1 in a fashion similar to that detected in endothelial cells.

Finally, we needed to evaluate the functional significance of muscle-specific receptors. This was accomplished by several means. Initially, we used adenoviruses expressing soluble Tie-2 and we evaluated the importance of Tie-2 in the pro-proliferative effects of Ang-1 on skeletal myoblasts. Our results indicate that Tie-2 receptors are essential for the promotion of myoblast proliferation by Ang-1. Our second approach was the use of retroviruses expressing Tie-2 siRNA to generate Tie-2 low skeletal myoblasts. Using these cells, we were able to show the importance of Tie-2 receptors in the late differentiation phases of skeletal myoblasts. Reduction of Tie-2 expression in Tie-2 low myoblasts resulted in attenuation of myoblast differentiation capacity as indicated by lower levels of p21, MyoD and myogenin expression (chapter 4 figure 19).

An incidental finding in chapter 4 is that the immortalized murine myoblast C2C12 cell line appears to express much lower levels of Tie-2 receptors compared with primary human and murine skeletal myoblasts. In a similar fashion, we have previously published a study in which we identified significant differences between primary murine and C2C12 myoblasts in terms of NADPH oxidase subunit

expression (237). These results indicate that investigators should be cautious when using C2C12 myoblasts when it comes to the issue of angiopoietins.

Second: We document for the first time that Ang-1 promotes skeletal muscle regeneration both *in-vitro* and *in-vivo*. For *in-vitro* experiments, we exposed skeletal myoblasts to exogenous Ang-1 or we infected these cells with adenoviruses expressing Ang-1 and assessed several parameters of the myogenesis program including myoblast proliferation, migration and differentiation. We showed in Figure 2 that Ang-1 but not Ang-2 promotes myoblast proliferation and that this response is mediated through Tie-2 receptors since expression of soluble Tie-2 receptors eliminated the pro-proliferative effect of Ang-1 on skeletal myoblasts. Myoblast proliferation is one of the initial and critical events in the regenerative process designed to replace necrotic muscle fibres with newly formed fibres. To evaluate the influence of Ang-1 on skeletal myoblast survival and apoptosis, we used serum deprivation as a stimulus to increase apoptosis and cell death in a fashion similar to that used in endothelial cells. As in the case of endothelial cells, Ang-1 strongly attenuated apoptosis and improved skeletal myoblast survival. Moreover, Ang-1 enhanced skeletal myoblast migration, another important process required for the regeneration program of skeletal muscle. These three effects (promotion of proliferation, survival and migration) of Ang-1 in skeletal myoblasts are quite similar to those described in endothelial cells. The exact cellular signaling pathways involved in mediating these effects were not identified, but we found that Ang-1 triggers a strong increase in the phosphorylation of ERK1/2, AKT, mTOR, FAK1 and paxillin in skeletal myoblasts (Figure 7). All these pathways are known to mediate the

biological effects of Ang-1 on endothelial cells (133, 177, 262). Accordingly, we propose that Ang-1 promotes several processes required for proper myogenesis program of skeletal myoblasts and that this effect is mediated through activation of multiple pathways including the PI-3 kinase/AKT, mTOR, ERK1/2 and integrin pathways.

To evaluate the influence of Ang-1 on the differentiation program of skeletal myoblasts, we used several approaches. We evaluated initially the influence of recombinant Ang-1 on p21, MyoD, myogenin, PGC1α, creatine kinase and myosin heavy chain expression. The expression of all these genes rose significantly after 24 hours of Ang-1 exposure (chapter 4, figure 4). To study the influence of Ang-1 over longer periods of differentiation (up to 7 days), we used adenoviruses expressing Ang-1 or GFP (control). Our results show that myoblasts expressing Ang-1 and undergoing differentiation expressed substantially higher levels of MyoD, myogenin, creatine kinase and myosin heavy chain than myoblasts expressing GFP. Our third set of experiments was designed to confirm that Ang-1 promotes the expression of myogenin, an important transcription factor involved in the expression of muscle-specific proteins. For these experiments, we transfected skeletal myoblasts with myogenin promoter along with expression vectors expressing Ang-1 and MyoD. In the absence of MyoD vector, Ang-1 was incapable of inducing myogenin promoter, however, when both Ang-1 and MyoD expression vectors were presented, Ang-1 was able to induce myogenin promoter to levels higher than those achieved with MyoD expression vector alone suggesting that Ang-1 activates pathways that work in concert with MyoD to augment myogenin expression and consequently muscle-

specific proteins. The nature of these pathways needs to be elucidated in future research. However, our own microarray study revealed that Ang-1 regulates the expression of several proteins that may have a direct influence on the differentiation of myoblasts. One of which is leptin which we also documented (chapter 3) to be induced by Ang-2 in cultured skeletal myoblasts. Other pathways that may also play important roles in the pro-differentiation effects of Ang-1 in skeletal myoblasts are FGF4 and the VEGF receptor NRP1 which are both significantly upregulated in response to Ang-1 exposure (chapter 4, table 2). One interesting finding shown in chapter 4 table 2 is that Ang-1 downregulated the expression of Hepatocyte Growth Factor (HGF) in skeletal myoblasts. Although HGF was suggested to be important in activating skeletal satellite cells, administration of HGF in mice and exposure of isolated satellite cells to HGF resulted in a significant inhibition of differentiation of these cells (233). These findings suggest that Ang-1 may promote myoblast differentiation in part through downregulation of HGF production.

To confirm the influence of Ang-1 on *in-vivo* muscle regenerative capacity, we conducted *in-viv*o experiments in which tibialis anterior muscle injury was first induced in mice by the injection of cardiotoxin. Four days after the injury, we administered adenoviruses expressing GFP (control) or Ang-1. The experiments were terminated after 10 or 14 days of the initiation of the injury and muscle morphology, vascular density, indices of muscle regeneration and contractility were measured. Our results show that Ang-1 administration significantly enhances muscle regeneration as indicated by the increase in the number of regenerated fibres, the proportion of fibres with more than one central nucleus, and by the significant

increase in the proportion of fibres expressing embryonic myosin heavy chain. Interestingly, Ang-1 did not influence muscle cross sectional areas of the recovering tibialis muscle indicating that Ang-1 promotes muscle regeneration but not hypertrophy. This finding suggests that Ang-1 does not cause the induction of growth factors that are known to induce skeletal muscle hypertrophy such as Insulin-like Growth Factor 1 (IGF-1). We actually tested the influence of Ang-1 on IGF-1 expression in both cultured human skeletal myoblasts and we also measured differences in IGF-1 expression in recovering tibialis anterior muscles receiving GFP vs. those receiving Ang-1 adenoviruses. Our experiments (unpublished observations) indicate that Ang-1 does not change IGF-1 expression.

One of the major findings of chapter 4 is that injection of Ad-Ang-1 viruses 4 days after the initiation of tibialis muscle injury by cardiotoxin resulted in complete recovery of tibialis muscle contractility while muscles receiving Ad-GFP viruses remained substantially weaker than un-injured muscles (chapter 4, figure 10). The exact mechanisms responsible for complete recovery of tibialis contractility in response to Ang-1 administration remain speculative but they are most likely due to increased regenerative capacity of satellite cells as documented in the *in-vitro* experiments. Another factor that may have played a major role in muscle force recovery is the increase in vascularisation of regenerating TA muscles which received Ad-Ang-1 injection. Thus, Ang-1 therapy appears to have improved both, skeletal muscle contractile performance and vascular density. It should be emphasized that we do not exclude possible effects of Ang-1 on processes such as excitation, excitation-contraction coupling and the contractile myofilaments that may have also

caused improvements of the contractile performance. Future studies using isolated muscle fibres should be conducted to evaluate the possibility of a direct effect of Ang-1 on muscle contractile processes. In summary, the main message of chapter 4 is that Ang-1 has a strong influence on the myogenesis program of skeletal satellite cells and that this effect when combined with enhanced survival of these cells and increased capillary formation leads to significant improvement of the regenerative capacity of injured skeletal muscles. Future research should be directed toward assessing the efficacy of Ang-1 therapy in pathologies where muscle regeneration is a major issue such as Duchenne Muscular Dystrophy.

CHAPTER 6

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APPENDIX