# ANGIOTENSIN II REGULATION OF SKELETAL MUSCLE GROWTH

# ANGIOTENSIN II REGULATION OF SKELETAL MUSCLE REGENERATION, GROWTH AND SATELLITE CELL FUNCTION

By

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## ABSTRACT

Local renin-angiotensin systems (RASs) have been described in many mammalian tissues. However, the role of angiotensin II (Ang II) in skeletal muscle is poorly understood with initial reports suggesting it may function to regulate overload-induced hypertrophy. Therefore, the purpose of this thesis was to 1) investigate the potential that adult skeletal muscle and muscle stem cells possess a local RAS. 2) Describe its role in regulating skeletal muscle regeneration and growth following injury and 3) demonstrate its capacity to regulate muscle stem cell activity and myogenesis. We report that cultured primary and C2C12 myoblasts and myotubes possess a local Ang II signalling system evidenced by the differential expression of angiotensinogen, angiotensin converting enzyme (ACE), and both angiotensin type 1 and 2 (AT1, AT2) receptors. Interestingly, myoblasts demonstrated the capacity to produce Ang II in spite of lacking renin expression. Furthermore, angiotensin receptors demonstrated differential localization with AT1 associated with actin filaments in proliferating myoblasts, and localized to the nucleus in differentiated myotubes. We also report that a local angiotensin system is present *in vivo* and responsive to myotrauma as cardiotoxin injection (to induce muscle injury) resulted in the increased staining intensity of angiotensinogen and AT1 during myogenesis with a progressive downregulation throughout the regenerative timecourse.

To investigate the effects of Ang II signalling blockade on muscle growth and regeneration we induced muscle injury in mice supplemented with captopril

(ACE inhibitor) or mice devoid of the AT1a receptor. Histological analysis revealed that ACE inhibition resulted in a decreased muscle fibre growth, increased proportion of small myofibres, an inability to accrete myonuclei and a robust hyperplasia of muscle fibres. Similarly, AT1a receptor ablation resulted in decreased muscle fibre growth following injury suggesting that these effects are receptor specific.

To investigate the mechanisms underlying these effects we assessed the role of Ang II in regulating muscle satellite cell function. *In vitro* experiments revealed that Ang II had the ability to regulate the early response of satellite cells to muscle injury by acting as a potent transcriptional activator of quiescent myoblasts and directing their subsequent migration. Furthermore, these migratory effects were mediated through an Ang II-induced increase in matrix metalloproteinase 2 (MMP2) content and reorganization of the actin cytoskeleton. Interestingly, Ang II may also participate in the fusion of myoblasts as captopril treatment suppressed the expression of markers of differentiation (myogenin) and maintained the expression of markers of proliferation (Pax7, Myf5). In agreement with this, IHC analysis revealed that ACE inhibition also induced a strong trend for a decrease in the proportion of myogenin positive cells following injury. Collectively, these results implicate the activation of local Ang II signalling system as a pleiotropic regulator of skeletal muscle growth.

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## ABBREVIATIONS

| | | |
|---|---|---|
| 4EBP1 | - | 4 Elongation binding protein 1 |
| ACE | - | Angiotenisn converting enzyme |
| ACE2 | - | Angiotenisn converting enzyme 2 |
| Ang I | - | Ang I |
| Ang II | - | Ang II |
| Ang III | - | Ang III |
| Ang IV | - | Ang IV |
| Ang 1-7 | - | Angiotenisn 1-7 |
| Ang 1-9 | - | Angiotenisn 1-9 |
| ANOVA | - | Analysis of variance |
| AP-1 | - | Activator protein-1 |
| ARB | - | Angiotensin receptor blocker |
| ARP 2/3 | - | Actin related protein 2/3 |
| AT1 | - | Angiotensin type 1 receptor |
| AT1a | - | Angiotensin type 1a receptor |
| AT2 | - | Angiotensin type 2 receptor |
| ATPase | - | Adenosine triphosphatase |
| ATRAP | - | Angiotensin receptor associated protein |
| β-Gal | - | β-Galactosidase |
| bFGF | - | Basic fibroblast growth factor |
| bHLH | - | Basic helix-loop-helix |
| CCR2 | - | Chemokine receptor 2 |
| CCR5 | - | Chemokine receptor 5 |
| CD45 | - | Cluster of differentiation 45 |
| ChIP | - | Chromatin immunoprecipitation |
| Con | - | Control |
| CREB | - | cAMP response element-binding protein |
| CSA | - | Cross-sectional area |
| CTX | - | Cardiotoxin |
| D | - | Deletion |
| DMD | - | Duchenne muscular dystrophy |

| | | |
|---|---|---|
| ECM | - | Extracellular matrix |
| EDL | - | Extensor digitorum longus |
| EGFR | - | Epidermal growth factor receptor |
| eMHC | - | Embryonic myosin heavy chain |
| FAK | - | Focal adhesion kinase |
| FBS | - | Foetal bovine serum |
| G1 | - | Gap 1 |
| G2 | - | Gap 2 |
| GCPR | - | G-coupled protein receptor |
| H&E | - | Hematoxylin and eosin |
| HGF | - | Hepatocyte growth factor |
| I | - | Insertion |
| IGF-1 | - | Insulin-like growth factor-1 |
| IHC | - | Immunohistochemistry |
| IL-4 | - | Interleukin-4 |
| IL-6 | - | Interleukin-6 |
| JAK | - | Janus kinase |
| LC-MS | - | Liquid chromatography-mass spectrometry |
| LDH | - | Lactate dehydrogenase |
| MAPK | - | Mitogen activated protein kinase |
| MHC | - | Myosin heavy chain |
| MMP | - | Matrix metalloproteinase |
| MRF | - | Myogenic regulatory factor |
| MRI | - | Magnetic resonance imaging |
| mRNA | - | messenger ribonucleic acid |
| NCAM | - | Neural cell adhesion molecule |
| NFATC2 | - | Nuclear factor of activated t-cells |
| NF-KB | - | Nuclear factor- KB |
| PDGF | - | Platelet derived growth factor |
| PFA | - | Paraformaldehyde |
| PI3K | - | Phosphoinositide 3-kinase |
| PKC | - | Protein kinase C |
| PLC-γ1 | - | Phospholipase C- γ1 |
| PLoS | - | *Public Library of Sciences* |

| | | |
|---|---|---|
| PTPase | - | Protein tyrosine phosphatase |
| qRT-PCR | - | Quantitative real time-polymerase chain reaction |
| RAS | - | Renin-angiotensin system |
| ROI | - | Reactive oxygen intermediates |
| S | - | Synthesis |
| S6K | - | S6 kinase |
| Sca1 | - | Stem cell antigen 1 |
| STAT | - | Signal transducers and activators of transcription |
| TA | - | Tibialis anterior |
| TGF-β | - | Transforming growth factor- β |
| TNF-α | - | Tumor necrosis factor-α |
| UI | - | Uninjured |
| VEGF | - | Vascular endothelial growth factor |
| VSMC | - | Vascular smooth muscle cell |
| WASP | - | Wiskott-Aldrich syndrome protein |

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## CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIP

### Chapter 2

***Publication***

Johnston, A.P.W., Baker, J., De Lisio, M. & Parise, G. Skeletal muscle myoblasts possess a stretch-responsive local angiotensin signaling system (2010). *Journal of the Renin-Angiotensin Aldosterone System.* In Press.

***Contribution***

The principle investigator of the study was GP. Experiments were conceived by APWJ and GP. *In vivo* experiments, myoblast isolation, cell culture, immunohistochemistry, nuclear/cytosolic preparation, western blotting and quantitative real time polymerase chain reactions (qRT-PCR) of proliferating and differentiating C2C12 and primary cells were conducted by APWJ. Mechanical stimulation and qRT-PCR of proliferating cells was conducted by MDL. Mechanical stimulation and qRT-PCR of differentiated cells was conducted by JB. Statistical analyses and manuscript preparation were completed by APWJ.

## Chapter 3

### *Publication*

Johnston, A.P.W., Baker, J., Bellamy, L.M., McKay, B.R., De Lisio, M. & Parise, G. Regulation of muscle satellite cell activation and chemotaxis by angiotensin II. In review at *PLoS One*.

### *Contribution*

The principle investigator of the study was GP. Experiments were conceived by APWJ and GP. Mouse breeding and maintenance was conducted by the McMaster Central Animal Facility. Cardiotoxin ejections were conducted by MDL and APWJ. Tissues were harvested by MDL and GP. LMB performed histological analysis. Methocellulose culture suspension, under agarose migration assays, transwell migration assays, checkerboard assays, invasion assays and coculture/flex migration assays were developed and conducted by APWJ and JB. Myf5-LacZ staining was conducted by JB. BRM conducted qRT-PCR analysis of the effect of Ang II on quiescent myoblasts. Phalloidin staining, gelatin zymography, propidium iodide, 7AAD and BrdU staining was conducted by APWJ. Flow cytometry analysis was conducted through collaboration with Dr. Borham. The manuscript was written by APWJ and edited by JB, LMB, BRM, MDL and GP.

## Chapter 4

### *Publication*

Johnston, A.P.W., Bellamy, L.M., De Lisio, M. & Parise, G. Captopril treatment induces hyperplasia and inhibits myonuclear addition following severe myotrauma. In submission at the *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.*

### *Contribution*

The principle investigator of the study was GP. Experiments were conceived by APWJ and GP. Mouse maintenance was conducted by the McMaster Central Animal Facility. Cardiotoxin ejections were conducted by MDL and APWJ. Tissues were harvested by MDL and GP. LMB performed histological analysis. LMB and APWJ performed qRT-PCR analysis. APWJ conducted IHC staining and analysis. The manuscript was written by APWJ and edited by MD, LMB and GP.

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 INTRODUCTION

Skeletal muscle is the largest organ in the body, the biggest depot of protein storage, the principal site of glucose disposal and has the ability to act as an endocrine, paracrine, and exocrine organ. Therefore, muscle not only has the capacity to regulate itself, but also serves to influence other cell types both intrinsic to muscle as well as through systemic means. Skeletal muscle also displays extensive plasticity with the innate ability to augment myofibre size in response to increased load or undergo extensive atrophy following prolonged disuse. Furthermore, skeletal muscle has a tremendous capacity to regenerate following injury. This is due to the fact that skeletal muscle also contains numerous stem cell populations including myogenic [1], endothelial [2], and mesenchymal [3]. Myogenic stem cells possess the capacity for extensive cell division and differentiation as well as self renewal or replenishment of the stem cell pool [4]. These processes are largely regulated by growth factors, but unfortunately many of the principal regulators remain undefined and require further identification.

## 1.2 OVERVIEW AND HISTORY OF THE SYSTEMIC RENIN-ANGIOTENSIN SYSTEM

It is has been over 100 years since Tigerstedt and Bergman first analyzed

the pressor effect of rabbit renal extracts [5]. They seminally discovered that blood extracted from the renal vein contained a vasoactive compound that when injected into nephrectomized animals induced a sustainable increase in blood pressure. This substance was aptly named renin. However, it wasn't until 1940 when both Page and Helmer [6] and Braun-Menéndez and colleagues [7] simultaneously described that renin acted enzymatically on a systemic compound resulting in the formation of what would eventually be termed angiotensin. Since these initial experiments research into the renin-angiotensin system (RAS) has grown at a rapid rate with many laboratories publishing countless investigations into the function, regulation, inhibition and propagation of this signaling cascade. In fact, over 21,135 peer reviewed manuscripts on the RAS have been published since 1960. In its classical interpretation, the RAS can be considered a systemic signaling system initiated by the release of renin from the kidney. Renin then acts on circulating angiotensinogen to form the inactive angiotensin I (Ang I). Ang I is enzymatically cleaved by the angiotensin convering enzyme (ACE) found predominantly on the luminal surface of lung endothelium to form active angiotensin II (Ang II; figure 1) [8]. The RAS has been a topic of intense investigation due to the integral role this system plays in blood pressure regulation and fluid homeostasis with newly described roles in cardiac cell growth, endothelial cell function and skeletal muscle adaptation. The following section will review the major components of the RAS with special attention to its regulatory function in local tissues as well as the systemic and local effects of

inhibition of this cascade.

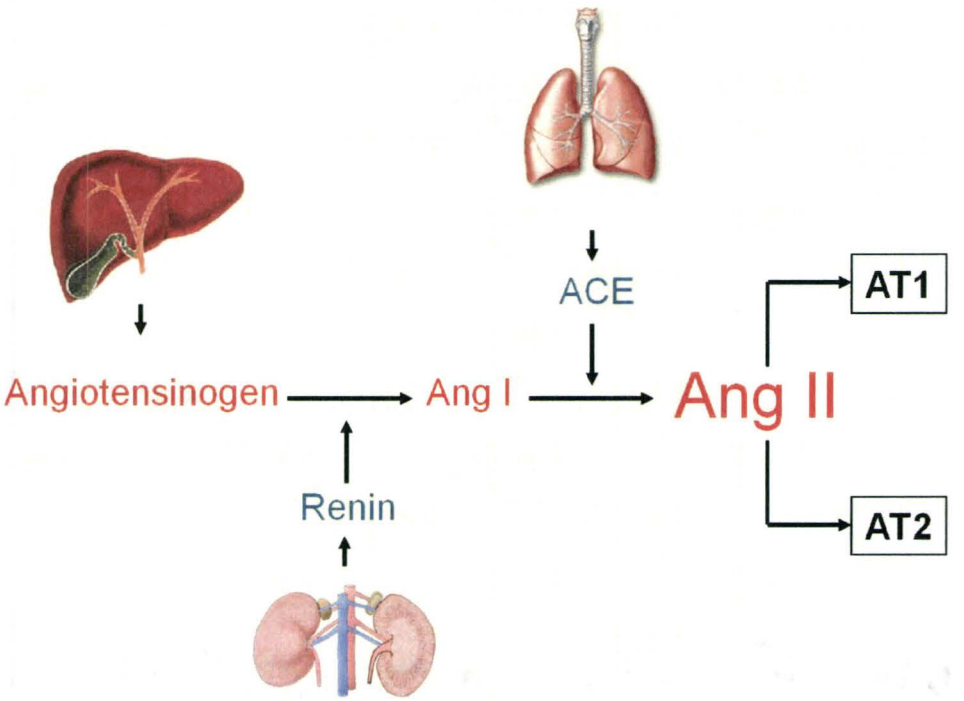


**Figure 1. Schematic diagram of the systemic RAS**. Renin is released by the kidney and acts on circulating angiotensinogen inducing the formation of Ang I. Ang I is subsequently cleaved by ACE catalyzing the formation of Ang II which has the capacity to bind two receptor subtypes, AT1 and AT2.

### 1.2.1 Renin, Angiotensinogen, ACE, Ang I

Renin is commonly considered the rate limiting enzyme in the production of Ang II and is therefore an integral mediator of this cascade. The primary source of renin production is renal juxtaglamerular cells and is secreted as an inactive zymogen called prorenin that requires cleavage enzymatically by the protease cathepsin B [9]. Renin is an aspartyl protease who's only known substrate is angiotensinogen and is therefore the primary enzyme regulating its conversion to Ang I [10]. The complexity of this system has recently been highlighted by the

discovery of a cell surface receptor with the ability to bind both renin and prorenin [11] and is termed the (pro)renin receptor. Interestingly, binding of renin to this receptor increases its enzymatic action while the binding of prorenin results in its ability to generate Ang I in the absence of the cleavage of the prosegment [11].

The liver appears to be the major source of circulating angiotensinogen secretion and extrahepatic angiotensinogen production is thought to be negligible [12]. Biochemically, the cleavage of angiotensinogen by renin or prorenin occurs between the amino acid residues 10 and 11 [13]. Interestingly, other enzymes such as cathepsin D have been demonstrated to possess "renin-like" enzymatic activity on angiotensinogen resulting in Ang I production [14]. However, during systemic functions, alternative renin-like enzymes are thought to play a minimal role. Many factors can regulate angiotensinogen production with higher plasma concentrations observed in patients with Cushing's syndrome, following glucocorticoid treatment or androgen treatment or in women who are pregnant or taking oral contraceptives [13]. Currently, angiotensinogen appears to act only as reservoir for Ang I and has little bioactivity.

Although Ang I contains the same 8 amino acid sequence as Ang II there is currently no known biological function for this decapeptide. Therefore, to become biologically active the ACE must cleave the C-terminal histidine – leucine residues resulting in the formation of Ang II [15]. ACE is also known as peptidyl-dipeptidase A and is a zinc metallopeptidase while functioning as the primary enzyme involved in the conversion of Ang II from Ang I [15]. ACE also indirectly

affects blood pressure by catalyzing the cleavage of bradykinin, thereby eliminating its vasodilatory effect [15]. This dual role for ACE in vasoactivity has made it an ideal candidate for pharmacological manipulation in the prevention of hypertension (discussed in section 1.2.3). ACE is comprised of an intracellular domain, a transmembrane domain and two extracellular domains consisting of the amino or N domain and the carboxy or C domain [16]. The 22 amino-acids located near the carboxy terminal domain acts as a transmembrane protein serving to anchor ACE to the cell surface [15]. ACE is primarily localized to neuro and absorptive epithelium and endothelial cells [17] with the capillary endothelial cells of the lungs catalyzing the majority of Ang II formation. On the surface of pulmonary endothelial cells ACE is positioned for optimal interaction with Ang I resulting in extremely efficient Ang II generation. For example, a single passage through pulmonary circulation is sufficient to convert all circulating Ang I into Ang II [18].

A gene polymorphism of a 287-base-pair insertion (I) has been identified in the ACE gene and the absence of this insertion (deletion – (D)) is associated with higher plasma ACE activity [19]. Many investigations have explored the association between the incidence and progression of cardiovascular disease to the ACE genotype; however, the results remain unequivocal [20]. Interestingly, recent investigations have made associations between the ACE genotype and physical performance. Specifically, homozygous expression of the D allele has been associated with enhanced training-related strength gains while homozygous

expression of the I allele has been linked with improved endurance performance [21, 22]. Although not fully substantiated, these reports are intriguing as they demonstrate the diverse importance of the RAS and earmark ACE as a gene polymorphism directly affecting physical capacity.

### 1.2.2 Angiotensin II and blood pressure regulation

Ang II is the primary end product of the RAS and is comprised of only 8 amino acids (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Although identified as a pleiotropic factor in many tissue types, Ang II is best known for its role in blood pressure regulation and fluid balance. The Ang II pressor response occurs rapidly (within 10-15s) by directly acting on atriolar vascular smooth muscle cells (VSMC) to induce their contraction subsequently increasing peripherial resistance and blood pressure [23]. Ang II chronically regulates blood pressure by acting directly on the kidney to signal renal sodium, $H_2O$ and bicarbonate resorption [24]. It also indirectly regulates blood pressure through stimulation of the adrenal gland to produce aldosterone or by acting on the central nervous system to stimulate the thirst response [24]. All of the known vasoregulatory actions of Ang II are signaled through the Ang II type 1 (AT1) receptor (please see section 1.2.3 for a detailed description of Ang II receptor signaling).

In circulation, Ang II is short-lived with an estimated half-life of ~1min in humans and may be completely removed following one pass through circulation [25]. However, little is known regarding the half-life of Ang II within individual tissues following uptake or due to local production. Systemic Ang II appears to

be primarily degraded in the kidneys by glomerular filtration, hydrolysis by renal tubule cells and subsequent reabsorption of the breakdown products [26, 27]. Ang II may also be hydrolyzed within the vasculature [28] or circulation; however, *in vitro* studies suggest that plasma Ang II breakdown is slow and is likely negligible in comparison to renal clearance [29]. Specifically, Ang II is broken down by a class of enzymes collectively termed "angiotensinases". Examples of these enzymes include trypsin, chymotrypsin, angiotensinogenase A, aminopeptidase and pepsin [30]. Following breakdown Ang II is metabolized into angiotensin III (Ang III) which can be further hydrolyzed into angiotensin IV(Ang IV), the functions of which are currently unknown but are currently being investigated [28].

### 1.2.3 Angiotensin receptors, signal transduction and growth effects

Classically, Ang II binds two cell surface receptor subtypes AT1 and the Ang II type 2 (AT2) receptor [30]. Both AT1 and AT2 are hetero-trimeric G-coupled protein receptors (GCPR) that span the plasma membrane seven times and like other GCPRs, AT1 undergoes internalization and recycling upon agonist stimulation [31]. Rodents express two isoforms of the AT1 receptor (AT1a and AT1b [32]) that share 94% sequence homology while the human genome contains only one AT1 receptor gene [33]. The AT1 receptor has been localized to many tissues throughout the body including the heart (epicardium, atrial and ventricular myocardium) [34], vasculature (aorta, pulmonary arteries, smooth muscle) kidneys and CNS [35]. Interestingly, recent reports have also

demonstrated the presence of AT1 receptors located within the nucleus of many cell types including VSMC [36] human endocardial endothelial cells [37], ventricular cardiomyocytes [38], and renal cells [39], the function of which is not completely understood.

This ubiquitous AT1 expression across organs and tissues led to the discovery that Ang II signaling has pleiotropic cellular effects independent of its role in the regulation of blood pressure and fluid balance. For example, AT1 mediated Ang II signaling has been demonstrated to increase the rate of protein synthesis, induce hypertrophy, and increase proliferation of VSMC [40] and cardiomyocytes [41, 42]. These trophic effects are induced through activating a plethora of signaling cascades including protein kinase C (PKC) [40], phospholipase C-γ1 (PLC-γ1) Src kinase, janus activator kinase-signal transducer and activator of transcription (JAK-STAT) signalling [43], mitogen activated protein kinase (MAPK), activator protein-1 (AP-1), epidermal growth factor receptor (EGFR), and other reactive oxygen species mediated pathways (for detailed review see [44]). These findings subsequently led to the discovery that Ang II signaling is a key mediator of hypertension, the progression of atherosclerosis, cardiac hypertrophy, remodeling and pathology [45].

Although much effort has been spent investigating the actions and molecular events governing AT1 signaling, very little is known regarding AT2 stimulation. AT2 is predominately expressed during fetal development but becomes rapidly downregulated following birth and has only been localized within adult brain,

heart, kidney and vascular endothelium [46]. Current evidence suggests that AT2 actions generally oppose those of AT1. For example, AT2 activation in cultured rat neonatal cardiomyocytes and fibroblasts inhibited the AT1 mediated hypertrophic response [47]. This is in agreement with the observation that stimulation of AT1 in neonatal cardiomyocytes results in enhanced survival while AT2 stimulation induces apoptosis [48]. Similar effects were also observed in VSMC and endothelial cells where stimulation of the AT2 receptor blocked AT1-mediated proliferation and growth [49, 50]. Although the underlying mechanisms governing these events are ill defined, there is evidence suggesting that AT2 stimulation results in activation of protein tyrosine phosphatase (PTPase) which subsequently inactivates the AT1 receptor and its downstream target of MAPK [51, 50, 52].

Ang II signaling is also an integral growth factor associated with adaptation to exercise and muscle overload. Numerous lines of evidence have demonstrated a link between Ang II and the angiogenic response. *In vitro* assays have demonstrated that Ang II promotes endothelial cell hypertrophy, proliferation [53] and capillary tube formation and upregulates the angiogenic hormone vascular endothelial growth factor (VEGF) [54]. This relationship has also been established *in vivo* by demonstrating that rats put on a high salt diet exhibit a decrease in skeletal muscle capillarization and Ang II production. However, exogenous infusion to maintain Ang II levels in spite of the high salt diet resultes in a rescue of angiogenesis and maintenance of capillarization to levels similar to

that of controls [55]. More recently, Ang II has been implicated in the process of exercise-induced angiogenesis. Capillarization can be achieved in skeletal muscle by chronic electrical stimulation or treadmill running. However, the increase in vessel density due to these interventions can be completely abolished by inhibiting Ang II signaling through treatment with captopril (an ACE inhibitor) or losartan (angiotensin receptor blocker (ARB)) during the exercise or electrical stimulation protocols [56, 57].

Based on the effects of Ang II on other myogenic lineages (i.e. VSMC, cardiomyocytes), the contribution of Ang II during skeletal muscle hypertrophy has been investigated using a model of muscle overload called synergist ablation. Gordon and colleagues [58] demonstrated that administration of an ACE inhibitor during a 28 day overload period resulted in a near complete abolishment of hypertrophy measured by total soleus muscle protein content. Confirming the role Ang II in this process, they were also able to rescue this defect by perfusing exogenous of Ang II into the soleus muscle. Moreover, similar results were obtained when losartan (ARB) was administered highlighting the role of AT1 signaling during muscle growth. These findings are further supported by Westercamp and colleagues [59] who demonstrated that ACE inhibition also abolished gains of muscle protein content and fibre cross-sectional area (CSA) using a similar overload model. They further demonstrated that these effects were potentially mediated by muscle satellite cells since no new muscle fibre nuclei were detected within soleus myofibres.

Although these studies demonstrate convincing evidence that Ang II plays a pivotal role in muscle hypertrophy, conflicting findings have been reported. For example, it has also been reported that infusion of Ang II into skeletal muscle resultes in a marked loss of skeletal muscle mass and a decreased expression of insulin-like growth factor-1 (IGF-1) [60]. Similarly, chronic infusion of Ang II into mice or treatment of C2C12 myoblasts resultes in decreased mitochondrial content, increased reactive oxygen species (ROS) production and increased intramuscular triglyceride content [61]. Therefore, further investigation is necessary to clarify the role of Ang II and the downstream signalling events regulating skeletal muscle growth, adaptation and atrophy.

### 1.2.4 Local renin-angiotensin systems

Although, the RAS has long been considered a systemic signaling cascade, our understanding of this system has expanded significantly and evidence suggests that this view is outdated. Recent reports have demonstrated the presence of "local" or "tissue" RAS systems that may function in concert or independent of the systemic RAS. These local systems are defined by the identification of some or all components of the RAS (angiotensinogen, renin, ACE, AT1, AT2) in individual tissues or organ systems and the ability to locally produce Ang II (for an extensive review of local RASs please refer to [62]). Local RASs have been indentified within many human tissues including: heart, nervous system, vasculature, reproductive tract, skin, digestive organs, sensory organs, lymphatic tissue, bone marrow and adipose tissue [62]. The best described local

RAS has been identified in cardiac tissue. Cardiomyocytes express a truncated (non-systemic) isoform of renin that increases following myocardial infarction and possesses the ability to absorb systemic renin from circulation [63]. Furthermore, the content of angiotensinogen, ACE, Ang I and Ang II [64] as well as both angiotensin receptors [65] have all been described within human heart. This local system becomes activated in response to mechanical stretch of cardiomyocytes which results in the the upregulation of virtually all of the RAS members [66] and functions to induce cardiac hypertrophy, remodeling and fibrosis [62].

Although our knowledge of local RAS has grown exponentially over the last 20 years, very few inquiries have probed for the presence of a local RAS in skeletal muscle and none have investigated its existence or function in the muscle stem cell compartment. This line of research is particularly difficult since there exists a number of cell types found in skeletal muscle that have already been identified as expressing components of a local RAS. Nevertheless, human skeletal muscle biopsies have been reported to possess ACE activity which does not correlate with serum ACE activity [67]. Furthermore, the AT1 and AT2 receptors have been localized throughout the skeletal muscle microvascular bed in rodents [68, 69], however, work in human muscle only confirms the presence of AT1 [70]. It has also been reported that whole skeletal muscle possesses the capacity for *de novo* Ang I and II synthesis that contributes to the circulating pool [71]. However, based on the available literature it is most appropriate to consider

the microvascular bed of skeletal muscle to be the site of Ang II production as no investigations have supplied definitive evidence that skeletal muscle cells independently produce Ang II [72].

### 1.2.5 Renin-angiotensin system inhibition

Hypertension is a chronic disease and one of the most commonly treated adult pathologies. Furthermore, hypertension is an epidemic within the elderly population with 70-85% of Canadians over the age of 70 years [73] and 60% of Americans over the age of 60 years [74] considered clinically hypertensive. Within the United States this results in an approximate cost of 37 billion dollars related to the cost of medication, office visits, and laboratory tests placing a significant economic burden on the health care system [75]. Consequently, aggressive pharmacological treatment of hypertension is a necessity and the most common approach involves drugs targeting *de novo* synthesis of Ang II through ACE inhibitors, or blockade of Ang II signalling, through ARBs [76]. Indeed, ACE inhibitors and ARBs represent the most widely prescribed class of anti-hypertensive medications [76]. These medications are moderately effective at reducing blood pressure with observed reductions of between 11/8 to 34.5/6.2 mmHg depending on the specific medication selected and the severity of hypertension [77].

Similarly, ACE inhibitors are also now widely prescribed as the first line of defence for the treatment of myocardial infarction, congestive heart failure [78] and atherosclerosis [79] and are utilized for slowing the progression of diabetic

and non-diabetic nephropathy [78]. Interestingly, some of the effects induced by this appear to occur irrespective of a decrease in blood pressure which has lead to much speculation as to their mechanism of action.

## 1.3 Overview of Skeletal muscle growth and regeneration

Remarkably, skeletal muscle has the innate capacity to efficiently regenerate from extensive damage. The skeletal muscle regenerative response primarily occurs in three distinct phases: 1) degeneration of damaged fibres, 2) repopulation of new muscle fibres and 3) growth and maturation of these fibres. Since muscle is post mitotic, it possess a depot of undifferentiated stem cells with the capacity to regenerate functional muscle fibres and repopulate the stem cell pool to allow for regeneration from subsequent bouts of trauma. Not surprisingly these stem cells termed "satellite cells" are also integral in the maintenance, growth and adaptation of skeletal muscle. This stem cell population engages in a series of events referred to as the "myogenic program" that consists of cellular activation, migration to the site of injury, numerous rounds of division, differentiation and subsequent fusion. The following section will review the phases of muscle generation and growth with special attention to the role of satellite cells, the myogenic program and cell chemotaxis.

### 1.3.1 Muscle degeneration

Upon irreparable damage to skeletal muscle myofibres a cascade of events is triggered that initiates the necrosis of damaged fibres. Experimentally, this can

be induced through various techniques including the injection of snake venom called cardiotoxin (CTX), the application of freeze/crush or forced downhill running [80]. This damage is measured quantitatively by serum analysis of proteins normally found in the cytosol such as creatine kinase [81, 82] or through histological analysis (i.e. hematoxylin and eosin (H&E) staining) to reveal muscle architecture. In humans the mechanisms governing the necrosis of muscle fibres are not completely understood; however, it is hypothesized that a loss of calcium homeostaisis due to sarcolemmal and sarcoplasmic reticulum disruption are significant factors [83]. The increased intracellular calcium is thought to activate calpains which cleave myofibrillar and extracellular proteins and subsequently induce necrosis of the myofibres [84].

Another important characteristic of muscle degeneration is the induction of inflammation. The inflammatory process serves two functions during regeneration: 1) to aid in the phagocytosis of apoptotic and necrotic fibres and 2) to participate in the healing and formation of new muscle fibres. This concept is highlighted by investigations demonstrating that depletion of monocytes at the site of muscle injury results in a delay and incomplete muscle regeneration *in vivo* [85]. The current literature suggests that muscle damage induces rapid activation of resident macrophages which subsequently induces the chemotaxis of circulating inflammatory cells to the site of injury [86]. Specifically, neutrophil accumulation has been observed within 1-6 hours following downhill running in humans [87]. This is followed by the recruitment of one main population of

monocytes that become inflammatory macrophages between 1-2 days following injury. Interestingly, these inflammatory macrophages undergo a phenotypic switch to anti-inflammatory macrophages following the phagocytosis of necrotic muscle fibres [85]. Furthermore, the secretion of factors from macrophages has been demonstrated to effect muscle stem cell growth and is therefore thought to be important in activating the muscle stem cell response [86].

### 1.3.2 Muscle satellite cells – indispensable regulators of regeneration and growth

The regeneration of skeletal muscle fibres following injury progresses through degeneration, inflammation and repair on a continuum such that there is considerable overlap between these phases. Following the primary inflammatory response to muscle injury, the principal cell population responsible for regenerating skeletal muscle fibres are satellite cells [4]. Their indispensable role in this process is highlighted by reports demonstrating that local irradiation and subsequent failure of satellite cell division functionally inhibits regeneration [88]. Furthermore, it is postulated that exhaustion of the satellite cell pool is the underlying cause in the pathology and progression of Duchenne muscular dystrophy (DMD), a disease characterized by continual muscle breakdown and repair [89].

Proper progression of satellite cells through the myogenic program is also obligatory for skeletal muscle growth and hypertrophy. This is supported by the myonuclear domain theory. This theory postulates that each muscle fibre nuclei

governs a finite volume of cytoplasm. Thus, in order for the fiber to grow, new nuclei must be added, leading to an increasing number of myonuclear domains. [90]. The necessity for satellite cell activity and nuclear addition during muscle hypertrophy is elegantly demonstrated by studies utilizing a synergist ablation model of overload as well as gamma irradiation to incapacitate the satellite cell pool. All of these studies [91-93] demonstrated that skeletal muscle hypertrophy was completely blocked in the absence satellite cell function. Furthermore, numerous lines of evidence suggest that this effect is not due to non-specific toxic effects on the muscle. Firstly, gamma irradiation of the same dose used in the aforementioned studies does not induce overt muscle damage as evidenced by a lack of an increase in serum creatine kinase following irradiation [94] while doses almost 10-fold higher are required to provoke fibre damage or necrosis [95]. Importantly, the transcriptional and translational machinery also does not appear to be effected as no differences were observed between groups in total mRNA, myogenin and mechano-growth factor expression as well as phospho-S6K (S6 kinase) and phosphor-4EBP1 (4 elongation binding protein 1) content in the acute phase following irradiation [91].

### 1.3.3 Satellite cell identification, distribution and characteristics

Skeletal muscle satellite cells were first described by Muaro in 1961 [1] who demonstrated, using electron micrographs, that a quiescent cell population resided between the basal lamina and the sarcolamma of the myofibre. Due to their location on the periphery of the fibre Mauro named these cells "satellite

cells". This seminal finding shed valuable light on the observation by Lewis and Lewis who described that skeletal muscle fibres appeared to increase in size and nuclear number without observable cell division [96]. Today, satellite cells are identified by numerous techniques, both anatomical and genetic; however the gold standard remains electron microscopy [80]. Regrettably, this means of identification is limited by the cost and restricted availability of equipment. Therefore, the use of standard immunohistochemical identification of cell surface and intracellular markers has proven effective in localizing and isolating satellite cells. Numerous markers have been reliably utilized such as Pax7, neural cell adhesion molecule (NCAM), MyoD, M-cadherin and syndacan among many others (reviewed in [4]). Unfortunately, the use of more than one marker is often necessary to indentify all satellite cells as they represent a heterogeneous pool that temporally upregulate and repress the expression of many of the aforementioned markers.

Interestingly, muscle stem cell content changes throughout the lifespan with satellite cells making up ~30% of all myonuclei in the neonate which decreases to ~ 4% in the adult and ~2% at old age [97]. There also appears to be a fibre-type specific distribution of satellite cells. For example, the soleus muscle primarily comprised of slow twitch fibres, has been demonstrated to possess a 2-3 fold higher satellite cell content than the extensor digitorum longus (EDL), which is primarily comprised of fast twitch fibres. Moreover, when compared to fast twitch fibres, a higher proportion of satellite cells are associated with slow twitch fibres

within the same muscle [98]. Satellite cells also appear localized in close association with capillaries [99] and neuromuscular junctions [100] suggesting a role for cellular cross-talk.

### 1.3.4 Myogenic specification and transcriptional regulation

Identification of the transcriptional networks orchestrating the events of the myogenic program has been extensively studied over the last 30 years. Rudnicki and colleagues [101, 102] have highlighted the essential role of the paired-box transcription factor, Pax7, for the specification of stem cells to the myogenic lineage. Indeed, Pax7 is specifically expressed in satellite cells and analysis of $Pax7^{-/-}$ mice demonstrated a complete absence of satellite cells, decreased body weight, inhibited growth and premature death [102]. Furthermore, the forced expression of Pax7 in $Sca1^{+}$ (stem cell antigen 1) $CD45^{+}$ (cluster of differentiation 45) stem cells allows their specification into the myogenic lineage [101]. However, the necessity of Pax7 expression for myogenic specification has been challenged by Oustanin and colleagues [103] who demonstrated that even though $Pax7^{-/-}$ mice have a reduced regenerative capacity, they still possess low numbers of satellite cells. Therefore, further investigation is needed to delineate the exact function and necessity of Pax7 during myogenic specialization and regeneration from injury.

Along with Pax7, a group of basic helix-loop-helix (bHLH) transcription factors termed the "myogenic regulatory factors" (MRFs) have been described as integral to both embryonic and postnatal skeletal muscle development and

growth and consist of Myf5, MyoD, myogenin and MRF4 [104]. During development, the upregulation of Myf5 and MyoD are necessary for specialization of stem cells to the muscle lineage and transgenic mice lacking both transcription factors do not develop skeletal muscle [104]. Furthermore, myogenin null mice also demonstrate a complete lack of muscle development due to the inability of muscle stem cells to differentiate and form multinucleated myofibres [105]. Conversely, mutation of MRF4 results in a variety of muscle phenotypes ranging from severe disruption to normal development and therefore requires further investigation to delineate its role. During postnatal development Myf5 and MyoD play important roles in satellite cell activation and proliferation while myogenin and MRF4 function primarily during myoblast differentiation and fusion (discussed herein) [80].

### 1.3.5 Satellite cell activation

Under basal conditions, satellite cells are maintained in a state of relative quiescence and require activation by intrinsic and environmental signals (figure 2). Morphologically, quiescent satellite cells display a high nuclear-to-cytoplasmic ratio with decreased organelle content and in comparison to the muscle fibre nuclei exhibit a smaller size and increased level of heterochromatin [4]. These observations are in agreement with findings that satellite cells, in the basal state, are mitotically quiescent and have decreased rates of transcription compared to myonuclei [106, 4]. Many investigations have attempted to define markers of quiescent satellite cells, however, due to the heterogeneous nature of

this pool no single marker has successfully been identified. Nevertheless, quiescent satellite cells have been reported to express Foxk1, are devoid of MRFs [4] and display high levels of nestin [107] and myostatin [108].

When satellite cells become activated, a number of intracellular and ultrastructural changes occur including an increase in size and organelle content as well as a reduction in the nuclear-cytoplasmic-ratio and heterchromatin content [4]. This phase is also marked by the rapid expression of the MRFs Myf5 and MyoD [109]. Once activated, satellite cells are considered "myoblasts" (Due to their movement from the "satellite" position) and are completely restricted to the myogenic lineage. At this time Myf5 and MyoD function to activate satellite cells by increasing the expression of myogenic mRNAs and genes necessary for cell cycle progression. For example, a recent chromatin immunoprecipitation analysis (ChIP) demonstrated that MyoD possesses tens of thousands of binding sites throughout the genome and has revealed its ability to acetylate histone 4A [110]. Therefore, MRFs function both at the genetic and epigenetic level. It has also been shown that there is a high level of redundancy between Myf5 and MyoD as deletion of either transcription factor results in the upregulation of the other and normal embryonic muscle development [104].

Although much is understood about the transcriptional networks that govern satellite cell activation, little is known regarding the upstream signaling events or the growth factors that initiate their response. This is partly due to methodological difficulties associated with satellite cell isolation and analysis. For

example, the process of satellite cell isolation, purification and culturing results in cellular activation and proliferation. Furthermore, most investigations analyzing satellite cell activation have measured proliferation rate as an inference to activation, while few direct measure have been implemented. In cell culture, myoblasts are anchorage dependent; therefore, the use of methocellulose to suspend cells in culture leads to induced quiescence and synchronization in G1 [111]. As a result, methocellulose suspension has become a useful tool to investigate issues of cell activation. To date, the best characterized activator of satellite cells is hepatocyte growth factor (HGF). It has been reported that HGF levels increase in muscle following injury and can stimulate the activation and proliferation of satellite cells *in vitro* and *in vivo* [112]. Results from recent investigations have led to a framework of HGF activation and signaling. It is thought that nitric oxide synthase is released from the basal lamina following injury or mechanical stretch which in turn synthesizes nitric oxide. Nitric oxide then has the ability to liberate HGF from extracellular heparan sulfate proteoglycans permitting its binding to the receptor c-met on the satellite cell surface [113].

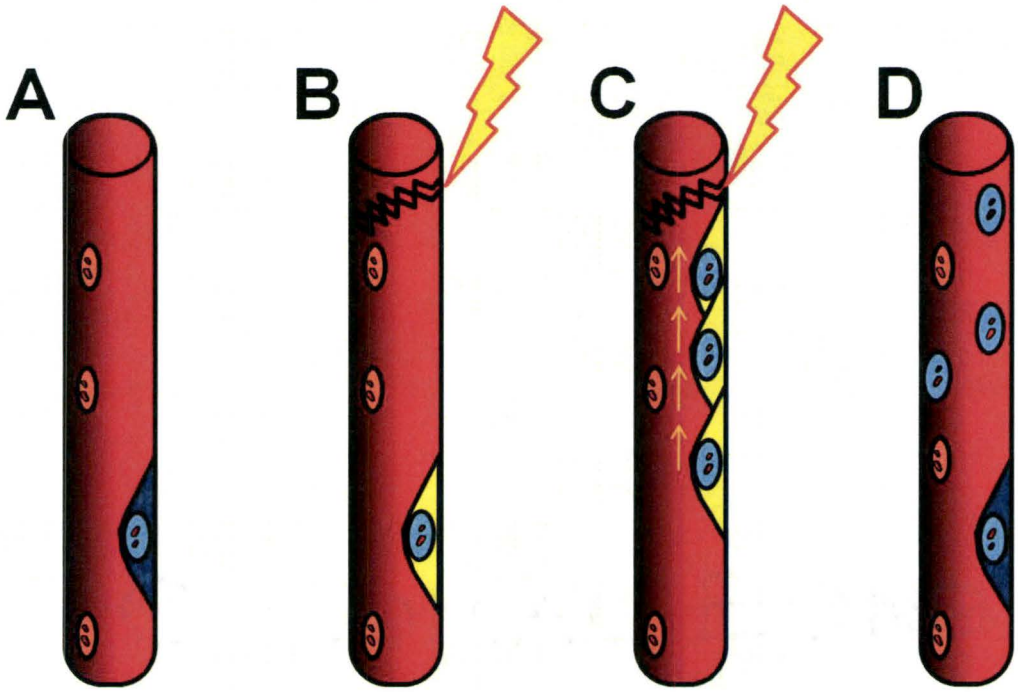


**Figure 2. Schematic diagram of the myogenic program**. A) Satellite cells normally sit on the periphery of the myofibre. B) In response to muscle damage, satellite cells become activated and are now considered myoblasts. C) These myoblasts will undergo rounds of division (proliferate) and migrate to the site of injury. D) Once at the injured area, myoblasts will align, terminally differentiate and fuse with the existing fibre (or together depending on the extent of damage) to restore skeletal muscle architecture.

### 1.3.6 Satellite cell proliferation

Once activated, expansion of the satellite cell pool (proliferation, figure 2) is necessary to allow for a sufficient number of cells to repair muscle damage. Growth factors play an important role in satellite cell proliferation with autocrine/paracrine signals as key regulators. The contribution of IGF-1 to satellite cell proliferation has been extensively studied. Using both animal models and *in vitro* experiments have demonstrated that exogenous IGF-1

treatment or genetic overexpression alters MRF expression, enhances myoblast proliferation and is necessary for overload-induced muscle hypertrophy [114-116]. Furthermore, work from our laboratory has demonstrated that these effects are likely conserved in humans as McKay and colleagues have demonstrated that IGF-1 is both localized within the satellite cell pool and its expression pattern is correlated with the activation of myogenic regulatory factors in response to eccentric damaging exercise [117].

Similarly, work from our laboratory and others have established a role for interleukin-6 (IL-6) signaling in myoblast proliferation. Serrano and colleagues have recently demonstrated that IL-6 knockout abrogates muscle fibre hypertrophy, myonuclear accretion and satellite cell proliferation *in vivo* and *in vitro* [118]. In agreement, McKay and colleagues demonstrated that IL-6 localized to satellite cells and correlated with the amplification of the satellite cell pool in humans following damaging exercise [119]. Although not specifically described in this review, a multitude of other growth factors have been reported to be pro-mitogenic for satellite cells such as: fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF, for an excellent review please see [4]). Similarly, a number of soluble factors are known to possess an inhibitory influence on satellite cell proliferation. Of the best described is the transforming growth factor-β (TGF-β) family of proteins. These proteins bind to cell surface receptors called SMADs, which function to inhibit myoblast proliferation and differentiation by silencing the

transcription of MRFs [120]. Recently, much research has investigated the TGF-β family member myostatin. The inhibitory effect of myostatin on satellite cell function is evident in rodent [121] and bovine [122] models of myostatin deletion, which present with extreme hypertrophy and hyperplasia. Furthermore, *in vitro* cell culture experiments demonstrate that myostatin induces its effect by inhibiting myoblast proliferation and differentiation by inactivating the retinoblastoma protein and subsequently inducing quiescence [123]. Due to the dynamic and heterogeneous nature of satellite cells, numerous growth factors have been shown to possess both mitogenic and inhibitory effects on satellite cell growth. For example, nuclear factor kappa-B (NFKB) and tumor necrosis factor-α (TNF-α) regulate many aspects of muscle regeneration, growth and proliferation [124]; however, uncontrolled signaling though these cascades is also associated with muscle atrophy, chronic inflammation and inhibited satellite cell expansion [125]. Therefore, these findings highlight the complexity of satellite cell regulation and function.

### 1.3.7 Myoblast fusion

Following adequate proliferation of the satellite cell pool, myoblasts must withdraw from the cell cycle, terminally differentiate and fuse together or to existing fibres (figure 2). This phase is marked by the increased expression of the MRFs, myogenin and MRF4 along with the cell cycle inhibitors p21 and p57 [109, 126, 127]. Consistent with this, Myf5 and MyoD undergo downregulation, however, the presence of MyoD is necessary for the transition of myoblasts from

proliferation into terminal differentiation. This is evidenced by the persistence of cell division under conditions of differentiation as well as the formation of small myofibres with few nuclei in MyoD$^{-/-}$ muscle cells [109]. Once myotubes have formed, they begin to express muscle specific proteins such as muscle creatine kinase and myosin heavy chain. Specifically, newly regenerated myofibres express the embryonic form of myosin heavy chain (eMHC) which is downregulated as the fibre matures, a process reminiscent of embryonic development [128].

Much of our knowledge regarding the temporal events and molecular machinery regulating the differentiation and fusion of myoblasts originates from studies investigating myogenesis in *Drosophila melanogaster*. These investigations have determined that myoblast fusion occurs in a specific, ordered sequence that begins with the determination of two separate types of myoblasts, "founder" cells and "fusion competent" cells [129]. Fusion competent cells recognize the founder cells, migrate to, and subsequently bind, initiating the fusion process [130]. Over the time course of these events, many adherence proteins are recruited to the site of cell-cell contact and function to reorganize the actin cytoskeleton to allow the process of fusion to occur. The cell mass is now referred to as a "syncytia", and grows in size and nuclear content through multiple rounds of fusion with additional fusion competent cells [130].

Although many of the structural and signaling proteins regulating *Drosophila* myoblast fusion have been identified, little research has identified or

validated the function of the mammalian homologues to these proteins. However, recent investigations suggest that mammalian myoblast fusion may be regulated by similar mechanisms. For example, mammalian post-natal muscle fibre formation also occurs in two distinct waves with the first consisting of myoblast-to-myoblast fusion and the formation of a "nascent" myotube followed by the second wave involving recruitment and fusion of additional myoblasts to the nascent myotube resulting in growth [131]. Interestingly, it has recently been demonstrated that myoblast-to-myoblast and myoblast-to-myotube fusion are discrete events at the molecular level and are regulated by different factors. Many growth factors have been shown to augment myoblast-to-myoblast fusion including IGF-1 [132], nitric oxide [133] and TGF-β [134]. Unfortunately, few cytokines have been identified to regulate myoblast-to-myotube fusion. Neverthe less, work from Pavlath and colleagues have demonstrated a central role for the nuclear factor of activated t-cells C2 (NFATC2) in the process. When stimulated to regenerate *in vivo,* NFATC2$^{-/-}$ muscle fibres formed normally but did not undergo substantial growth following formation. Similarly, cultured NFATC2$^{-/-}$ myoblasts were able to efficiently form nascent myotubes but were unable to accrete new nuclei resulting in small immature fibres [135]. In a follow up study, it was discovered that the downstream target of NFATC2 during the second wave of myoblast fusion was interleukin-4 (IL-4), the knockout of which also resulted in small immature fibre formation in culture [136]. NFATC2 can be regulated by a number of upstream factors which subsequently identify them as regulators of

mononuclear addition. These factors include prostaglandin $F_{2\alpha}$ [137], growth hormone [138] and potentially the c-src pathway [139]. Collectively these findings demonstrate that NFATC2 plays a pivotal role in regulating myonuclear accretion and therefore represents a novel therapeutic target for manipulation during muscle wasting diseases

1.3.8 Myoblast migration

Although satellite cell migration is not officially considered part of the myogenic program, it is a necessary component of muscle growth and repair. Since satellite cell quantity is low and distribution is sparse, muscle injury activates satellite cells distal to the injured site (figure 2). Therefore satellite cells must be able to "detect" muscle damage and possess the cellular machinery to migrate great distances to sites of injury. Albeit rare, under certain conditions it has been demonstrated that satellite cells can even migrate through the basal lamina to adjacent fibres [140].

Initial studies, using electron microscopy analysis of focal muscle injury, have shown that satellite cell migration precedes appreciable expansion of the pool with proliferation occurring at the site of injury [140]. Moreover, localized injury causes a gradient in satellite cell number extending along the fibre from the site to injury to the distal end of the muscle suggesting that even very far reaching cells migrate to the injured area [140]. Therefore, it appears that the early response to muscle injury consists of satellite cell activation and migration. Unfortunately, the molecular mechanisms and signaling cascades governing

myoblast migration are poorly defined.

Two forms of cellular migration exist, 1) chemokinesis, analogous to stochastic movement and 2) chemotaxis, analogous to directed homing. Although the function of chemokinesis is poorly defined, cell chemotaxis has been extensively investigated, primarily in the context of cancer metastasis. Fortunately, it is thought that many of the mechanisms regulating cancer metastasis are thought to also dictate the chemotaxis of many other cell types (for an excellent review please see [141]). To date, cell chemotaxis is best described as a cyclic process initiated by the binding of growth factors initiating pro-migratory signaling cascades. These cell surface receptors can be found evenly distributed on the cell membrane or concentrated at the leading edge of the migrating cell as in the case of the chemokine receptors 2 and 5 [142]. The initial chemotactic response for a cell is to become "polarized" (shape and molecular structures at the front and back of the cell are different) and begin to extend actin-rich protrusions [143]. The formation of these protrusions are absolutely necessary and can be in the form of large fan like structures called lamellipodia and smaller spike-like structures called filopodia [144]. Lamellipodia are ideally shaped for active chemotactic movement along the plasma membrane while filopodia serve to sense the local environment. The lamellipodial extensions then become stabilized by anchorage to the extra-cellular matrix (ECM) and function as traction sites as the cell undergoes actinomyosin contraction and is propelled forward. Finally, the rear of the cell becomes

detached and new lamellipodia are formed as the cell advances forward.

A number of intracellular proteins have been identified as being important for cell polarity and chemotaxis. Rac, Cdc42, Wiskott-Aldrich syndrome protein (WASP) and actin-related protein 2/3 (Arp2/3) have been identified as essential for the formation of lamellipodial projections and cell polarity [141]. Similarly, integrin proteins act as cellular "feet" attaching the lamellipodia to the ECM by linking to intracellular actin via adaptor proteins (i.e. Arp1/2, WASP). Integrins also function as signaling proteins, inducing the self propagation of pro-migratory signals [144]. Detachment of the cell from the ECM at the trailing end is facilitated by a group of proteases called matrix-metalloproteinases (MMPs). This large group of proteins consists of enzymes capable of degrading numerous types of collagen, gelatin, lamin, elastin and fibronectin [145]. They also function to degrade the ECM at the leading edge to make space for the ever expanding cell mass. Therefore, MMPs play an important role in numerous aspects of cell motility.

With regards to satellite cell chemotaxis, a handful of chemoattractive growth factors have been identified including IL-4 [136], IGF-1, basic fibroblast growth factor (bFGF) [146] and HGF [147]. Interestingly, recent investigations have implicated MMP activity as a major regulator of myoblast motility. El Fahime and colleagues [148] reported that skeletal muscle and C2C12 myoblasts predominantly expressed MMP2 and when over-expressed, demonstrate a significant enhancement in migratory capacity following *in vivo* injection.

Conversely, pharmacological inhibition of MMP2 impaired *in vivo* myoblast migration following injection.

Clinically, the enhancement of myoblast chemotaxis to the site of injury is a critical component for cell transplantation therapy. Myoblast transplantation into skeletal muscle has recently been considered a potential therapy for DMD. However, these treatments have had little success attributable to low survival, inadequate engraftment and poor migration from the site of injection [149]. However, a recent investigation by Wang and colleagues [149] demonstrated that co-injection of myoblasts along with MMP1 significantly improved engraftment and increased the number of dystrophin+ fibres in a mouse model of muscular dystrophy. Therefore, further research is needed to elucidate the role on MMPs and the soluble factors regulating their expression, processing and activity in the context of myoblast migration.

## 1.4 Purpose of Thesis

The research experiments conducted and described in this thesis were designed to analyze the role of Ang II signalling in skeletal muscle regeneration and growth. On the basis that skeletal muscle is largely intrinsically regulated, we first hypothesized that skeletal muscle and muscle satellite cells express a functioning local RAS consisting of the expression of the precursor peptide angiotensinogen, Ang II receptors and the ability to produce Ang II. We then aimed to identify the contribution of this local RAS to skeletal muscle

regeneration and growth induced by CTX-induced injury in mice undergoing pharmacological inhibition of Ang II production (using an ACE inhibitor) or mice devoid of the AT1a receptor. Therefore, we quantified the regenerative and growth responses using histological measures of newly formed fibres, fibre growth and accretion of nuclei. We also examined the *in vivo* activation of this local skeletal muscle RAS in regenerating and growing fibres. Since proper muscle satellite cell function is necessary for both regeneration from injury and growth, we examined the *in vivo* effect of ACE inhibition on MRF gene expression and satellite cell content. Finally, to further assay the contribution of Ang II signalling in regulating satellite cell function we analyzed the effect of Ang II treatment or loss of function on satellite cell activation, migration, proliferation *in vitro*. Collectively, the results provided within this thesis describe, in depth, the functions of Ang II in regulating muscle stem cell function, skeletal muscle regeneration and subsequent growth using *in vivo* and *in vitro* models.

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**Chapter 2: Manuscript 1**

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# Skeletal muscle myoblasts possess a stretch-responsive local angiotensin signaling system

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Running title: muscle stem cells and angiotensin

**Abstract:**

**Introduction**. A paucity of information exists regarding the presence of local renin-angiotensin system (RAS) in skeletal muscle and associated muscle stem cells. **Methods**. Skeletal muscle and muscle stem cells were isolated from C57Bl/6 mice and examined for the presence of a local RAS using qRT-PCR, IHC, western blotting and LC-MS. Furthermore, the effect of mechanical stimulation on RAS member gene expression was analyzed. **Results**. Whole skeletal muscle, primary myoblasts and C2C12 derived myoblasts and myotubes differentially expressed members of the RAS including angiotensinogen, ACE, AT1 and AT2. Renin transcripts were never detected, however, mRNA for the "renin-like" enzyme cathepsin D was observed and angiotensin I and II were identified in cell culture supernatants from proliferating myoblasts. AT1 appeared to co-localize with polymerized actin filaments in proliferating myoblasts and was primarily found in the nucleus of terminally differentiated myotubes. Furthermore, mechanical stretch of proliferating and differentiating C2C12 cells differentially induced mRNA expression of angiotensinogen, AT1 and AT2. **Conclusion**. Proliferating and differentiated muscle stem cells possess a local stress responsive RAS *in vitro*. The precise function of a local RAS in myoblasts remains unknown, however, evidence presented here suggests that angiotensin II may be a regulator a regulator of skeletal muscle myoblasts.

## Introduction

The renin-angiotensin system (RAS) is a potent regulator of blood pressure and fluid homeostasis. Angiotensin II (Ang II), the end product of the RAS, is synthesized through enzymatic cleavage of angiotensiogen by renin to form Ang I, which is subsequently cleaved by angiotensin converting enzyme (ACE)(1). Recently, the notion that Ang II only arises through systemic sources has been challenged by detailed descriptions of local RASs in tissues such as lymphatic, digestive, adipose as well as vascular and cardiac tissue (2). Local RASs are characterized by tissue-specific expression of RAS family members, *de novo* Ang II synthesis and the potential to function independently or in concert with the systemic RAS (2). Regrettably, the precise function of local RASs in peripheral tissues is not fully understood.

Mechanical stretch has been shown to be a potent activator of local RASs. In cultured cardiac myocytes, mechanical stimulation induced an upregulation of renin, angiotensinogen and ACE gene expression while also inducing the secretion of Ang II (3). Similarly, mechanical stimulation via pressure overload of cardiac myocytes, induced the upregulation of RAS family member expression *in vivo* and is hypothesized to mediate left ventricular hypertrophy (4). Evidence of a similar stretch-responsive RAS has been reported in mesangial cells. Mechanical stretch of cultured mesangial cells induced a significant upreglation in AT1 receptor mRNA and Ang II binding while also increasing angiotensinogen mRNA and Ang II secretion (5)

Indirect evidence for a local RAS in skeletal muscle is accumulating. Ang II has been shown to be necessary for synergist ablation- induced compensatory hypertrophy (6), and that a lack of Ang II in muscle attenuated the addition of new myonuclei (7). In spite of these results, it is not clear whether the effect of ACE inhibition, utilized in these experiments, was due to local or systemic blockade of Ang II. Although it has recently been reported that skeletal muscle possesses ACE activity (8, 9) as well as *de novo* Ang I and II synthesis (10), it is unclear if Ang II production occurs in skeletal muscle itself or through the associated microvascular bed (11). To date, it is unknown whether a local RAS exists in mature skeletal muscle fibres and myogenic stem cells (satellite cells). Moreover, it is unknown whether a local RAS is responsive to mechanical stimulation similar to other myogenic lineages such as cardiac myocytes.

**Methods**

*Animals*. Ten week-old male C57Bl/6 mice were maintained on 12 hour light:dark cycles and provided food and water *ad libitum*. Animals were euthanized by cervical dislocation and the tibialis anterior (TA) muscles were excised, snap frozen in liquid nitrogen and stored at -80°C. All procedures were conducted according to guidelines established by the Canadian Council on Animal Care with ethics approval from the McMaster University Research Ethics Board.

*Cell culture*. Primary myoblasts were isolated from the hind-limbs of wild-type C57Bl/6 mice as detailed previously (12). Briefly, satellite cells were isolated via

collagenase/dispase digestion of muscle followed by purification from fibroblasts through pre-plating. Experiments were only conducted after myoblasts had reached 99% purity. Cells were cultured at 37°C in 5% $CO_2$ in either proliferation media (PM; F-10 supplemented with 20% FBS, 1% penicillin-streptomycin and 2.5ng/ml of bFGF) or subsequently induced to differentiate in fusion media (FM; DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin). C2C12 myoblasts were grown at 37°C in 5% $CO_2$ in either growth media (GM; DMEM supplemented with 10% FBS and 1% penicillin-streptomycin), FM, or serum free DMEM when indicated.

*Immunohistochemistry.* Proliferating and terminally differentiated primary and C2C12 myoblasts were grown on gelatin-coated glass coverslips in PM, GM or FM respectively. Media was aspirated and coverslips were washed with PBS, fixed in acetone for 10 min (for proliferating cells) or 4% PFA for 5 min and dehydrated in either ice cold 100% EtOH (for terminally differentiated myotubes) or ice cold acetone (co-staining) then again washed in PBS for 3x5min. Coverslips were blocked for 1.5h with 5% goat serum and 0.2% sodium azide in PBS followed by overnight incubation with primary antibodies for AT1 or AT2 at 1:100 dilution (SC-1173, SC-9040 respectively, Santa Cruz Biotechnologies, Santa Cruz, CA USA) or with simultaneous incubation with anti-AT1 and TRITC-conjugated phalloidin (2µg/ml, Sigma-Aldrich, Canada cat#1951) at 4°C in 2% goat serum. Coverslips where then washed for 3x5min in PBST followed by incubation with goat anti-rabbit texas red or Alexa 488 secondary antibodies

(Molecular Probes, Invitrogen Canada). After 3x5min of washing in PBS, cells were counterstained with DAPI and coverslipped with mounting medium (Dako Canada) and visualized on a Nikon Eclipse 90i.

*RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction*. RNA was isolated from the tibialis anterior muscle, proliferating C2C12 and primary myoblasts, differentiating C2C12 and primary myotubes, or stretched proliferating and differentiating C2C12 cells using a combination of the TRIzol (Invitrogen) and the RNeasy method as per the manufactures instructions (Qiagen Sciences, USA). 1000ng of total RNA from each sample was then reverse transcribed in 20 µL reactions according to the manufacturer's instructions (Applied Biosystems High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA) and stored at -20°C. Quantitative RT-PCR (qRT-PCR) reactions were carried out using a Stratagene Mx3000P real-time PCR System (Stratagene, USA) using Stratagene MxPro QPCR Software Version 3.00 (Stratagene, USA). Fold changes in gene expression were calculated using the delta-delta Ct method (13). Primer sequences of genes analyzed were: AT1: for-ACAGTGATATTGGTGTTCTCAATGAAA rev-CCATTGTCCACCCGATGAA, AT2: for-CAGAATTACCCGTGACCAAG rev- TAAACACACTGCGGAGCTT, angiotensinogen: for- GAGGCAAATCTGAGCAACATTG rev-GAGTTCGAGGAGGATGCTATTGA, ACE: for-GTTCGTGGAAGAGTATGACCG rev-CCATTGAGCTTGGCGAGCTTG, renin: for-TCTCTGGGCACTCTTGTTGCTCTG rev-ATACGTCCCATTCAGCACTGAGCC,

cathepsin D: for-ATCTTGGGCATGGGCTACC rev-GGCTGGACACCTTCTCACAA, and ribosomal protein L32: for-TCCACAATGTCAAGGAGCTG rev- ACTCATTTTCTTCGCTGCGT.

*Mechanical stretch.* Actively growing C2C12 myoblasts or terminally differentiated myotubes were cultured on type I collagen-coated flexible-bottom plates (flex-cell plates; (Flexcell International, McKeesport, PA, USA) in GM or FM respectively. Proliferating cells were allowed to adhere for 12h while myotubes were induced to fuse for 7 days prior to mechanical stimulation. The stretch protocol consisted of cyclic strain at 0.1 Hz (8s of stretch alternated with 2s of rest) of a 20% strain for a period of 4 hours (14). Stretch was applied using the FX-4000 Tension Plus (Flexcell International, McKeesport, PA, USA), a vacuum stretch apparatus coupled to computer software.

*Liquid chromatography-mass spectrometry (LC-MS).* C2C12 myoblasts were grown for 24 hours in DMEM without FBS and the supernatants were collected for LC-MS analysis. Solid phase extraction (SPE) cartridges (Phenomenex StrataX, 10 mg) were washed with 1 mL of methanol and 1 mL of water. 20 uL of phosphoric acid was added to 1 mL of the sample. After loading, the cartridge was washed with 1 mL 20% acetonitrile and the sample eluted with 1 mL 80% acetonitrile. The eluant was dried and reconstituted in 100 µL 10% acetonitrile 0.1% formic acid. Samples were then run using an Agilent Poroshell 300SB-C18 2.1 x 75 mm column. Eluants were A: 1% formic acid, B: 1% formic acid in acetonitrile, with the following radient program: Initial A:B 95:5, hold 0.5 minutes,

3 minutes, A:B 60:40, 4 minutes, A:B 20:80. The following transitions were monitored: angiotensin II: 524→110 (45 V), angiotensin I, 433→110 (30 V) and Leucine enkephalin (internal standard) 556→120 (45 V).

*Nuclear/cytosolic protein fraction isolation and western blotting.* Nuclear and cytosolic protein fractions of terminally differentiated C2C12 cells were isolated using a commercially available kit (Pierce cat#78833) according to the manufactures instructions. Protein content was determined by Bradford Assay (Thermo Fisher Scientific, USA) and loaded (10% of total protein for AT1 and AT2 or 10 ng for LDH) into a 10% polyacrylamide gel and resolved for 90 min at 100 V. Gels were transferred overnight at 50 V to PVDF membranes, and equal loading was ensured through ponceau staining. Membranes were blocked in 5% skim milk in TBST for 2 h then incubated in primary antibodies (AT1 (1:500), AT2 (1:400) or LDH (1:2000, Cell Signalling cat#2012) for 2 h followed by 3x15 min of washing in 5% skim milk in TBST. Membranes were then incubated in HRP conjugated secondary antibody (1:25000 for AT1, AT2 and 1:10000 for LDH) followed by 5x10 min washing in TBST and reaction with Pierce SuperSignal substrate (Pierce cat#34075). Bands were visualized using the Alpha Innotech FluroChem SP and quantified using Alphaease software.

*Statistical analysis.* Data was statistically analyzed using SigmaStat 3.1.0 analysis software (Systat, SPSS Inc., USA). Proliferation/differentiation mRNA expression was assessed by one-way ANOVA followed by Tukey's post hoc test.

Western blots and mRNA expression from mechanically stimulated cells were analyzed by t-tests. Statistical significance was accepted at $P<0.05$.

**Results**

*Adult skeletal muscle, myoblasts and differentiated myotubes express a local angiotensin signaling system.* To examine if skeletal muscle fibres expressed members of the RAS, mRNA was isolated from the TA of C57Bl/6 mice. qRT-PCR analysis confirmed the expression of many RAS members including: angiotensinogen, ACE, AT1 and AT2 (data not shown). Interestingly, the expression of renin was not detected despite probing with three sets of primers. As whole skeletal muscle contains many cell types (i.e. muscle cells, myoblasts, fibroblasts, endothelial cells), we wanted to investigate if RAS member expression was localized within actively proliferating or differentiating myogenic progenitor cells. Therefore, we analyzed the expression of RAS members in two myogenic lineages: isolated primary myoblasts and C2C12 cells. In agreement with our *in vivo* findings, both proliferating primary and C2C12 myoblasts expressed mRNA transcripts for angiotensinogen, AT1 and AT2 (figure 1). When growth arrested and induced to differentiate, RAS member genes were also detected and demonstrated a differential expression pattern. For example, both Ang II receptors demonstrated similar expression until terminal differentiation (day7 for C2C12 cells, day3 for primary myoblasts due to differing differentiation kinetics) when AT1 increased significantly. Similarly, angiotensinogen

significantly increased during differentiation when compared to proliferation (figure 1). Importantly, primary and C2C12 cells demonstrated similar gene expression patterning in both proliferating and differentiated cells. In further agreement with the *in vivo* analysis, renin transcripts were not expressed in either myoblasts or myotubes derived from primary or C2C12 cells, however, the expression of cathepsin D, an enzyme that is reported to possess "renin-like" enzymatic action on angiotensinogen (15), was detected in proliferating myoblasts and was significantly upregulated during differentiation (figure 1). Similarly, the ACE was expressed in proliferating C2C12 myoblasts and significantly increased upon differentiation (figure 1) while it was only detected in terminally differentiated primay myotubes (data not shown). We also probed for members of the "extended" RAS including ACE2 and the MAS receptor but mRNA transcripts were not detected using multiple primer sets (data not shown).

*AT1 and AT2 demonstrate differential localization within muscle progenitors and differentiated myotubes.* To confirm our qRT-PCR findings, we assessed primary myoblasts/myotubes and C2C12 cells at the protein level using IHC and western blotting. We observed robust staining for AT1 and AT2 receptors in cultured primary and C2C12 myoblasts as well as differentiated myotubes (figure 2, 3). In proliferating cells, AT1 appeared to be predominately localized to cytoskeletal, filamentis proteins while AT2 demonstrated a more diffuse expression pattern. Based on the appearance of AT1 expression in both proliferating primary and C2C12 cells we conducted IHC co-staining to further define its location within the

cell. Interestingly, co-staining of AT1 and the actin marker, phalloidin, demonstrated many areas of co-localization within the cell (figure 4) suggesting that AT1 colocalized to cytoskeletal proteins. Furthermore, staining of terminally differentiated myotubes revealed that AT1 also appeared to be localized within the myonucleus (figure 3). To confirm this observation we isolated nuclear and cytosolic fractions from terminally differentiated C2C12 cells. Indeed, western blotting for the AT1 receptor revealed that it was primarily expressed within a nuclear enriched fraction as a ~500% increase was observed in comparison to the cytosolic fraction (figure 3c). Interestingly, blotting also demonstrated that AT2 only appeared to be localized within the cytosol (figure 3f). The purity of the nuclear/cytosolic preparation was confirmed by probing for the cytosolic specific marker LDH which only demonstrated expression within the cytosol (data not shown).

*Cultured myogenic progenitor cells secrete angiotensinogens.* To define myoblasts as possessing a local RAS, the cells must possess the ability to independently produce Ang II. Therefore, we assayed the ability of muscle myoblasts to produce angiotensins. In comparison to DMEM alone, LC-MS analysis of the cell culture supernatant of proliferating C2C12 cells revealed that myoblasts possess the ability to produce both Ang I and Ang II in culture within the 1.0-2.0 $ng \cdot ml^{-1}$ range (figure 2e, f).

*Mechanical stretch alters the expression of genes associated with the RAS.* As the expression of RAS members appeared to be stimulated by mechanical

stretch in other local RASs (4, 5, 16) we subjected proliferating myoblasts and differentiated myotubes to cyclic mechanical strain for 4 h. Mechanical stretch of both proliferating and differentiated C2C12 cells induced significant alterations in RAS member gene expression. In proliferating cells, angiotensinogen and both Ang II receptor subtypes demonstrated a significant upregulation 8 h post-stretch, while no effect was observed on ACE (figure 5 a-d) or cathepsin D expression (data not shown). Interestingly, 4 h of mechanical stretch induced differing effects on RAS member gene expression in terminally differentiated myotubes. Angiotensinogen demonstrated an early, significant increase in expression (4 h post, figure 5g) while the Ang II receptors demonstrated a reciprocal expression in response to mechanical stretch with AT1 downregulated at 8 h post stretch (figure 5e) while AT2 was upregulated 4 h post-stretch (figure 5f). Conversely, ACE expression was not altered by mechanical stretch in terminally differentiated myotubes (figure 5h)

**Discussion**

The present manuscript describes the first definitive evidence for a local angiotensin signaling system in isolated primary and C2C12 cells. Recent investigations have provided initial evidence for the presence of such a system. For example, Qi and colleagues (17), using radioligand binding, demonstrated that human skeletal muscle myoblasts possess AT1 and AT2 receptors with AT1 dominantly expressed. Furthermore, Mori and colleagues (18) reported that

proliferating C2C12 cells possess ACE activity. However, the presence of other RAS members, or the ability of these cells to independently produce Ang II, was not assessed in these manuscripts. Additionally, there is a complete lack of data identifying a function for a local RAS in skeletal muscle. Although recent advances in the literature have delineated many of the transcriptional networks orchestrating muscle stem cell function, the upstream signaling events that induce these networks are largely undefined. The current manuscript provides evidence implicating a role for a local angiotensin system in regulating proliferating and differentiated muscle stem cells.

Our results indicate that muscle stem cells appear to contain a local angiotensin signaling system with the ability to secrete Ang I and II *in vitro*. Other local systems have been described to function in concert with the systemic RAS (2), however, it is unknown whether the local RAS in skeletal muscle functions independently or in conjunction with the systemic RAS *in vivo*. Interestingly, C2C12 myoblasts were shown to possess the ability to secrete Ang I and II despite a lack of renin. This finding suggests that other enzymes function with renin-like enzymatic activity on angiotensinogen since this precursor peptide was identified in proliferating and differentiating cells. One candidate enzyme that demonstrated robust expression in proliferating and differentiating cells was cathepsin D. Cathepsin D has previously been shown to act on angiotensinogen (15) and is involved in the synthesis of Ang II in vascular smooth muscle cells (19) as well as adipose tissue (20).

The differential localization of AT1 and AT2 receptors in proliferating and differentiated cells suggests receptor specific function. Much recent literature has focused on the function of AT1 signaling as it is thought to be the major receptor subtype involved in regulating Ang II action on vascular smooth muscle cells (VSMC) vasoconstriction, proliferation and hypertrophy (21) as well as cardiac myocyte hypertrophy (22). An increasing number of reports have demonstrated the presence and localization of nuclear AT1 receptors. Recent investigations observed AT1 in the nucleus of human VSMC (23), human endocardial endothelial cells (24), ventricular cardiomyocytes (25), hepatocytes (26) and renal cells (27). Although there is a paucity of information regarding the function of nuclear AT1 receptors, initial reports suggest a role in regulating cellular proliferation (28). Furthermore, Cook and colleagues (28) observed that targeting AT1 to the nucleus by a novel fusion protein resulted in the activation of p38MAPK and subsequent downstream activation of CREB (cAMP response element-binding protein). The function of nuclear AT1 receptors in muscle cells is completely unknown but since skeletal muscle is terminally differentiated, signaling of Ang II through AT1 would not participate in cell division. Future investigations are needed to delineate its cellular function. IHC staining of angiotensin receptors in proliferating muscle stem cells also revealed differential localization in the cell. In comparison to AT2, which demonstrated a heterogeneous staining pattern, AT1 co-localized with the actin cytoskeleton in many areas. Although other cell surface receptors such as epidermal growth

factor receptor have been demonstrated to associate with the actin cytoskeleton and is an actin-binding protein (29, 30), to our knowledge this is the first description of localized AT1 with actin filaments in muscle stem cells. Interestingly, IHC staining also revealed areas where AT1 and actin did not associate but were in close proximity suggesting that AT1 may also co-localize with cytoskeletal adaptor proteins (31). It should also be noted that although AT1 and actin demonstrated IHC co-localization, it is unknown whether there is physical association between these proteins through the cell membrane or if other proteins are required for this association. Along these lines, recent reports have suggested a role for caveolar lipid rafts in associating cell surface AT1 with intracellular actin filaments through the protein, filamin (32). This AT1-caviolin relationship has been implicated in the regulation of tyrosine phosphorylation, reactive oxygen species (ROS) generation and signaling as well as VSMC growth and hypertrophy (32). At present, the functional significance of this AT1-actin association in muscle stem cells is unknown. In other myogenic lineages, Ang II signaling through AT1 has been shown to induce actin filament and cytoskeletal reorganization (33, 34) and consequently influence cellular migration and chemotaxis of endothelial cells (35), retinal microvascular pericytes (36), and VSMC (37), however, such a relationship has not been described in muscle stem cells.

In agreement with data from cardiomyocytes (4, 16) mechanical stimulation of proliferating myoblasts and differentiated myotubes activated the

local angiotensin signaling system in these cells. Mechanical stretch is a major component of skeletal muscle contraction and a significant regulator of muscle stem cell function. *In vitro,* stretch of muscle stem cells using a similar system has been demonstrated to activate other autocrine/paracrine signaling cascades (14), increase cell proliferation (38) and inhibit differentiation (39). Therefore, one could speculate that a local skeletal muscle and muscle stem cell angiotensin signaling system could become activated in response to muscle contraction *in vivo*. Interestingly, myoblasts and myotubes responded differently to mechanical stimulation. Of particular interest is the differential response of AT1 expression in myoblasts (increasing expression) versus myotubes (decreasing expression). Since we demonstrate that in terminally differentiated myotubes AT1 is primarily localized to the nucleus, our results suggest that mechanical stimulation transiently decreases nuclear mRNA production, the physiological consequences of which remain unknown. Ang II signaling through the AT1 receptor has been shown to be essential for the growth associated with overload-induced muscle hypertrophy (6), as well as the addition of myonuclei (7). Functionally, *in vivo* overload and *in vitro* mechanical stretch represent different models as the latter lacks metabolic perturbation and therefore may elicit differing responses. Furthermore, changes in mRNA may not directly affect cellular function as other factors such as receptor localization (membrane/nucleus) (28), association with antagonizing proteins (40), or activity of AT2 signaling (41) have also been demonstrated to regulate this system. It should also be noted that the optimal

duration and intensity of mechanical stimulation required for maximal activation of this system is unknown and manipulation of these factors could potentially yield differing results. However, additional experiments in our lab using 20 h of mechanical stretch demonstrated similar results with regards to changes in mRNA expression (data not shown).

Although it is not clear how blockade of Ang II production/signaling regulates muscle growth, it is reasonable to assume that muscle stem cells and post-mitotic fibres may be regulated, at least in part, by local Ang II. Ang II signaling through AT1 could potentially regulate skeletal muscle growth through numerous mechanisms including enhancing muscle stem cell function (such as activation, chemotaxis, proliferation or differentiation) as well as affecting protein synthetic rate of post-mitotic fibres as AT1 receptors were localized to both myoblasts and differentiated myotubes. Along these lines Ang II has been demonstrated to increase the proliferation and protein synthetic rate of other myogenic cell types such as VSMC (42, 43) and cardiomyocytes (44, 45). Interestingly, exogenous Ang II treatment of differentiated skeletal myotubes led to a 50% inhibition of protein synthesis (46) and protein degredation (47) *in vitro*. These incongruent results suggest although necessary for overload-induced skeletal muscle hypertrophy, Ang II may function in a "hormetic" fashion with chronic treatment resulting in net protein loss.

**Conclusion**

In summary, skeletal muscle derived stem cells possess a local angiotensin signaling system capable of producing Ang II despite the lack of renin. Interestingly, the AT1 receptor was primarily expressed in the nucleus of differentiated myotubes and co-localized with actin filaments in proliferating cells. Furthermore, cyclic mechanical stimulation of both proliferating and differentiating muscle precursors differentially activated this system. Taken together, these results suggest a regulatory role of a local angiotensin signaling system in proliferating and differentiated muscle stem cells which merits further investigation.

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**Figure Legends.**

**Figure 1. Muscle stem cells and differentiated myotubes express members of the RAS system.** qRT-PCR analysis of mRNA expression AT1 (A), AT2 (B), cathepsin D (C) angiotensinogen (D) and ACE (E) in proliferating (Pro) primary and C2C12 myoblasts and during 1, 3, and 7d of differentiation into myotubes. *indicates a significant difference ($p \leq 0.05$) from (Pro). Note: ACE expression was also observed in terminally differentiated primary myotubes (d3-diff).

**Figure 2. Proliferating primary and C2C12 myoblasts express angiotensin receptors and secrete angiotensins.** IHC staining of AT1 (A, B) and AT2 (C, D) in proliferating primary and C2C12 myoblasts. LC-MS analysis of Ang I (E) and Ang II (F) in cell culture supernatants of proliferating C2C12 myoblasts.

**Figure 3. Differentiated myotubes demonstrate nuclear localization of the AT1 receptor.** IHC staining of AT1 (A, B) and AT2 (D, E) in terminally differentiated primary and C2C12 myotubes. Note the presence of nuclear localized AT1 receptors. Representative western blots of AT1 (C) and AT2 (F) in (n)uclear and (c)ytosolic fractions of terminally differentiated C2C12 myotubes. *indicates a significant difference ($p \leq 0.05$) from cytosolic.

**Figure 4. The AT1 receptor co-localizes with polymerized actin filaments.** IHC staining of DAPI (A), phalloidin (B) AT1 (C) and overlay (D) in proliferating C2C12 myoblasts. Note the areas of co-localization indicated by arrows.

**Figure 5. Mechanical stretch alters the expression of RAS members in muscle cells**. qRT-PCR analysis of AT1 (A, E), AT2 (B, F), angiotensinogen (C, G) and ACE (D, H) mRNA expression in proliferating (A-D) and terminally differentiated (E-H) C2C12 cells in response to 4h of cyclic mechanical strain. * indicates a significant difference ($p \leq 0.05$) from control, unstretched cells.

## Figures.

## Figure 1.

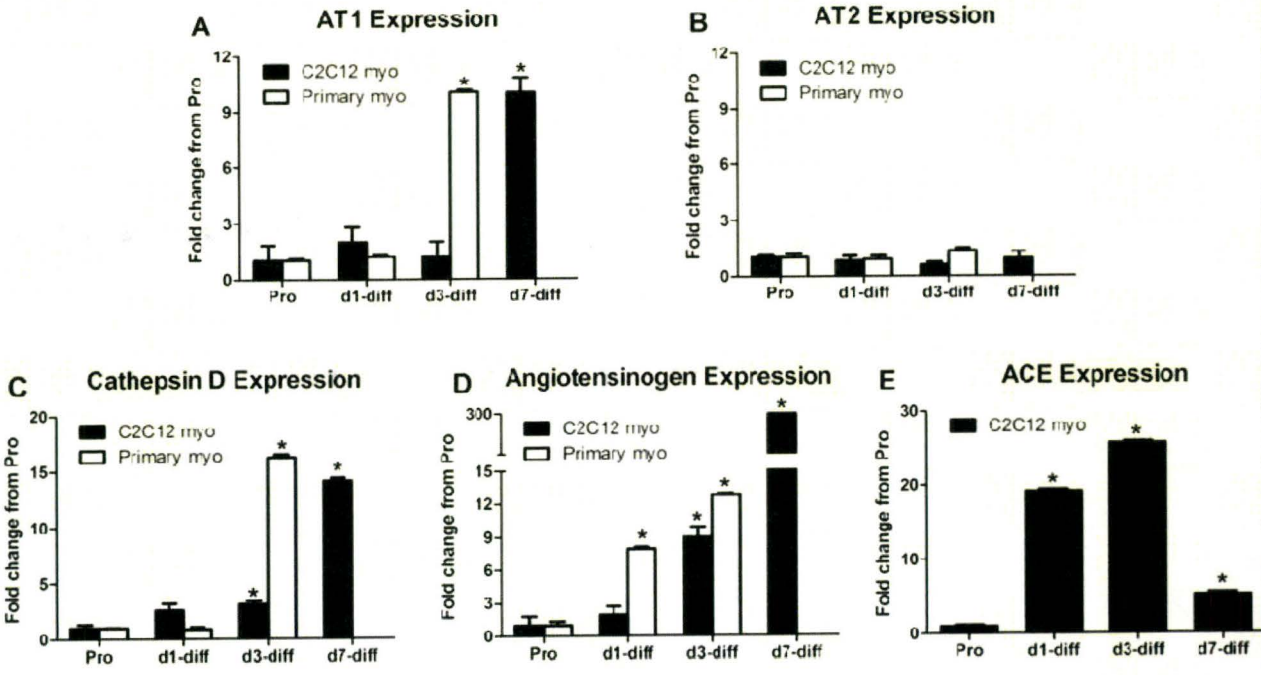
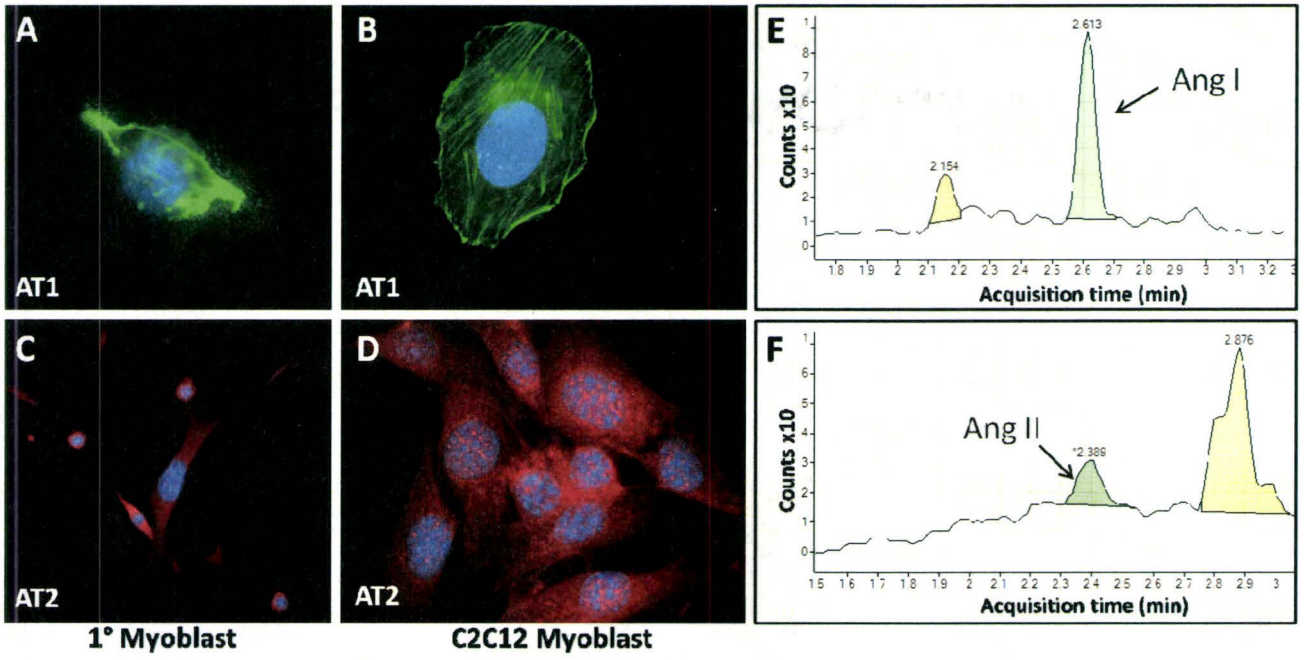


**Figure 2.**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B
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C
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Ang I
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F
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Ang II
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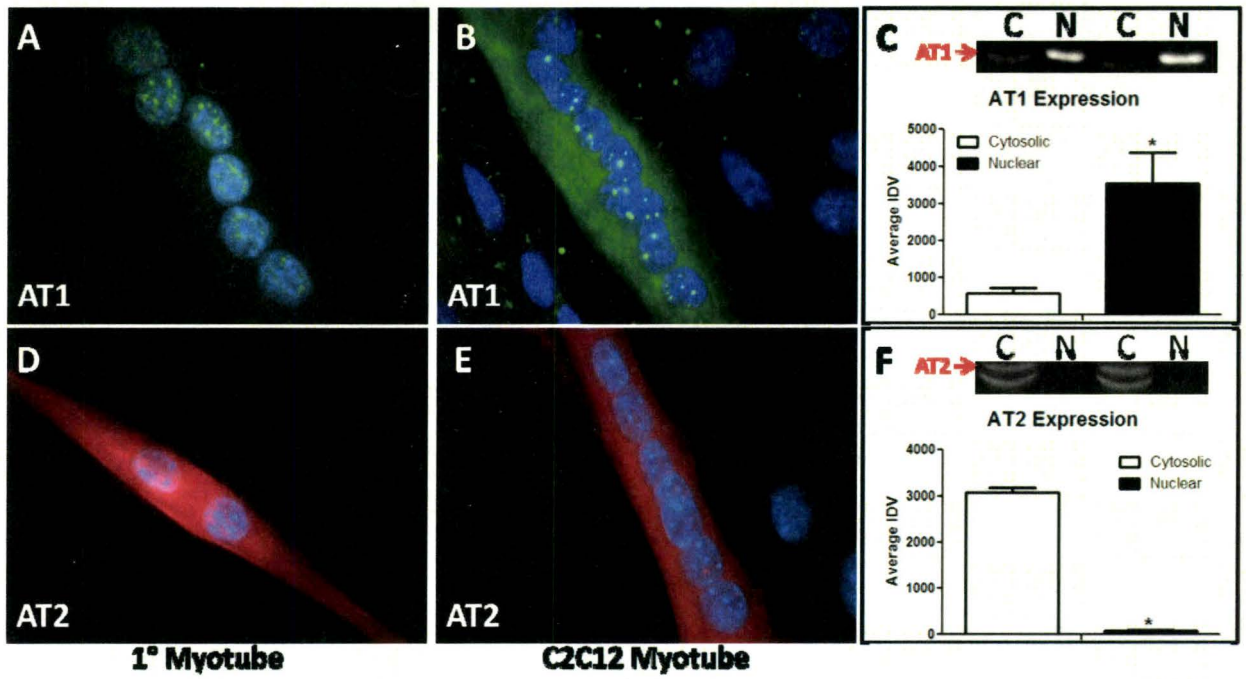
Counts x10
Acquisition time (min)

**Figure 3.**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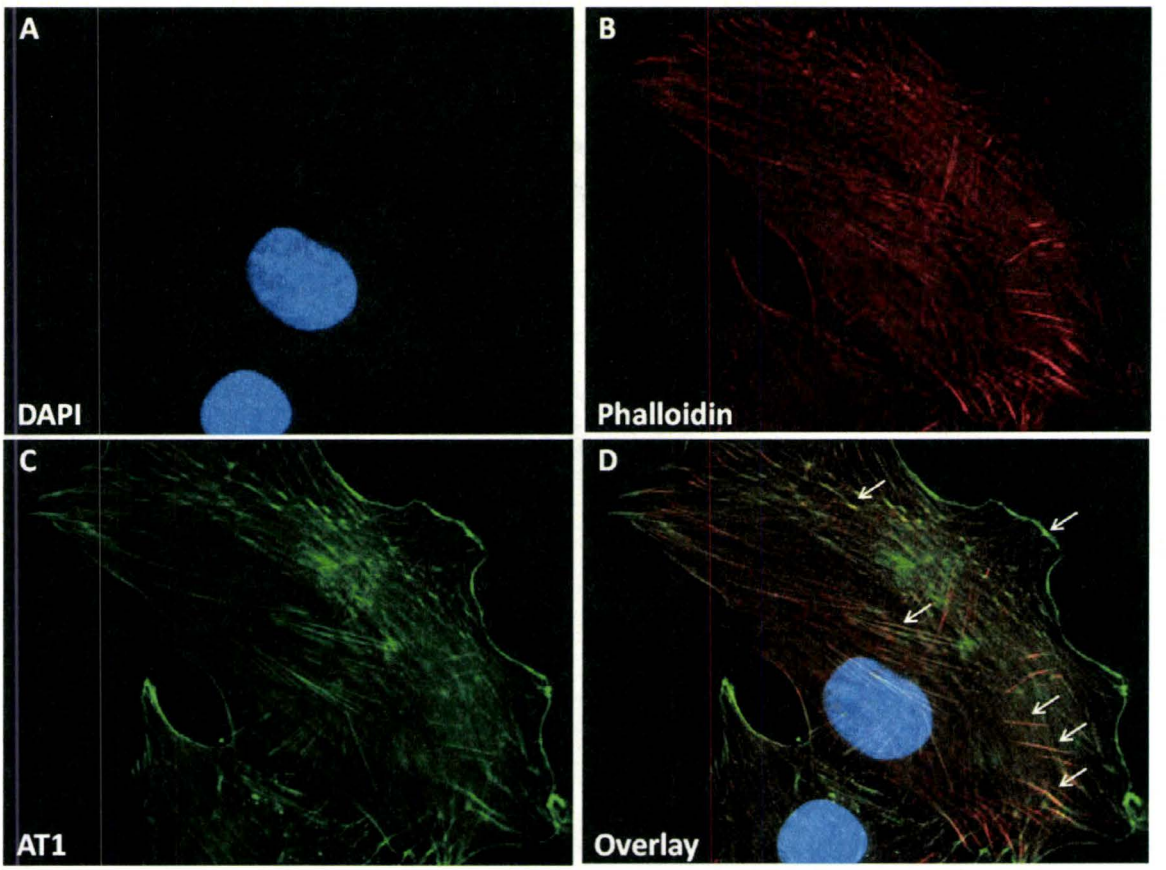
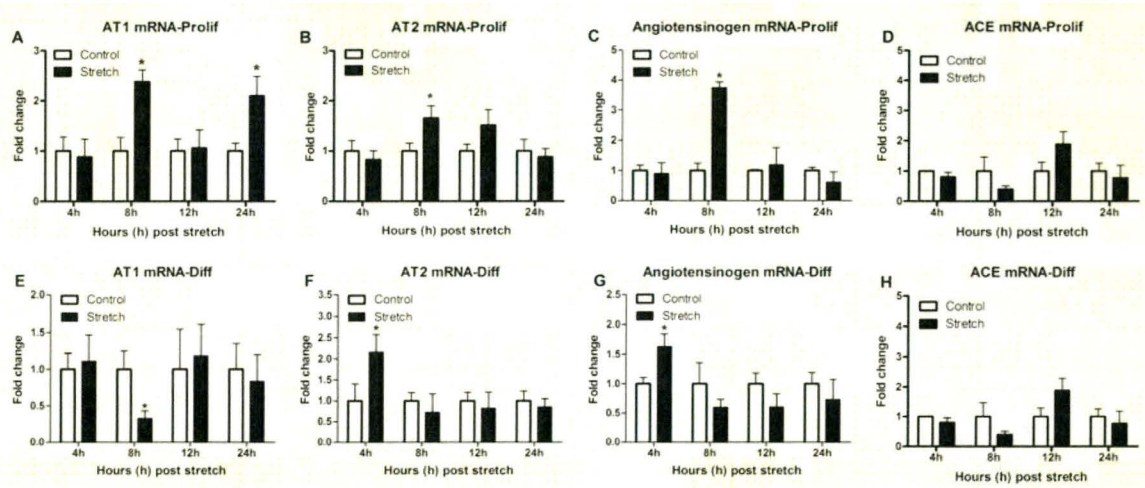


## Figure 5.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B
AT2 mRNA-Prolif
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Angiotensinogen mRNA-Prolif
D
ACE mRNA-Prolif
E
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AT2 mRNA-Diff
G
Angiotensinogen mRNA-Diff
H
ACE mRNA-Diff
Control
Stretch
Fold change
Hours (h) post stretch
4h
8h
12h
24h

## Chapter 3: Manuscript 2

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# Regulation of Muscle Satellite Cell Activation and Chemotaxis by Angiotensin II

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**Abstract**

The role of angiotensin II (Ang II) in skeletal muscle is poorly understood. We report that pharmacological inhibition of Ang II signaling or ablation of the AT1a receptor significantly impaired skeletal muscle growth following myotrauma, *in vivo,* likely due to impaired satellite cell activation and chemotaxis. *In vitro* experiments demonstrated that Ang II treatment activated quiescent myoblasts as evidenced by the upregulation of myogenic regulatory factors, increased number of β-gal+, Myf5-LacZ myoblasts and the acquisition of cellular motility. Furthermore, exogenous treatment with Ang II significantly increased the chemotactic capacity of C2C12 and primary cells while $AT1a^{-/-}$ myoblasts demonstrated a severe impairment in basal migration and were not responsive to Ang II treatment. Additionally, Ang II interacted with myoblasts in a paracrine-mediated fashion as 4h of cyclic mechanical stimulation resulted in Ang II-induced migration of cocultured myoblasts. Ang II-induced chemotaxis appeared to be regulated by multiple mechanisms including reorganization of the actin cytoskeleton and augmentation of MMP2 activity. Collectively, these results highlight a novel role for Ang II and ACE inhibitors in the regulation of skeletal muscle growth and satellite cell function.

## Introduction

Skeletal muscle is composed of post-mitotic, multinucleated fibres. Subsequently, growth and regeneration from injury is dependent on a population of muscle stem cells, referred to as "satellite cells", that are maintained in a state of quiescence under basal conditions. However, satellite cells can become activated in response to intrinsic and environmental cues associated with damage and overuse [1], a process characterized by the increased expression of myogenic regulatory factors such as MyoD and Myf5 [2] and immediate early genes such as cfos [3]. Once activated, satellite cells migrate to the site of injury, proliferate and subsequently differentiate and fuse to restore skeletal muscle architecture in a process referred to as the myogenic program [4, 5]. Although much is understood about the transcriptional networks governing the myogenic program, little is known regarding the upstream signals or soluble factors influencing moygenic regulatory factor expression and satellite cell function.

Specifically, there is a paucity of information regarding the factors that induce the activation of satellite cells with hepatocyte growth factor being the only reliably identified cytokine [6, 7]. Similarly, the temporal kinetics, soluble factors, or signaling cascades regulating satellite cell migration are poorly understood. Indeed, chemotaxis is integral to repair and growth of skeletal muscle as satellite cells are required to migrate great distances to sites of myotrauma, and properly align to undergo differentiation and fusion. Interestingly, hepatocyte growth factor

signaling has also been implicated in myoblast chemotaxis [8] suggesting a link between satellite cell activation and cellular motility.

Angiotensin II (Ang II) has been extensively studied in the context of its vaso-regulatory properties and the pharmacological inhibition of Ang II signaling to reduce blood pressure represents the most widely-prescribed anti-hypertensive therapy [9]. However, localized tissue renin-angiotensin systems (RAS) have been identified suggesting that Ang II may have wide ranging effects in addition to its systemic role in vasoregulation. For example, Ang II is now known to influence such diverse processes as cell proliferation, hypertrophy [10, 11] and migration [12-14]. Cultured skeletal muscle myoblasts and myotubes possess a local Ang II signaling system [15]; however, its function remains poorly understood. Importantly, it was reported that inhibition of Ang II signaling resulted in near complete attenuation of skeletal muscle hypertrophy in a model of synergist ablation [16, 17], suggesting that Ang II may regulate skeletal muscle hypertrophy. Regrettably, the precise role of a local RAS in skeletal muscle regeneration, growth and maintenance remains largely unknown.

The purpose of this investigation was to assess the role of Ang II in regulating the growth and repair of skeletal muscle, *in vivo,* as well as myoblast function *in vitro*. In this manuscript we report that the inhibition of Ang II signaling through captopril treatment or ablation of the Ang II type Ia receptor (AT1a) resulted in a significant impairment in skeletal muscle growth following cardiotoxin (CTX)-induced injury. Furthermore, *in vitro* experiments indicated that

Ang II regulates the early satellite cell response as exogenous treatment of quiescent myoblasts with Ang II resulted in an upregulation of myogenic regulatory factor expression indicating enhanced activation, as well as an increased chemotactic capacity attributable to signaling through AT1. Ang II-induced migration occurred through reorganization of the intracellular actin cytoskeleton and enhanced matrix metalloproteinase-2 (MMP2) activity. We also report that Ang II can function in a paracrine fashion signaling neighboring myoblasts to migrate in a coculture environment. Collectively, these results identify a novel role for Ang II in the regulation of skeletal muscle growth and muscle stem cell function. Furthermore, these results suggest that the widely prescribed anti-hypertensive drug, captopril, may have adverse affects on skeletal muscle growth and repair.

**Methods**

*Animals/experimental procedures*. Ten-week-old C57Bl/6 mice (study-1, n=10 per group) and fourteen-week-old AT1a$^{-/-}$ mice and aged matched C57Bl/6 controls (study-2, n=4 per group) (Jackson laboratories, USA) were utilized. Study-1 C57Bl/6 mice were supplemented with either normal drinking water or captopril (0.5mg/mL, Sigma, Canada) treated drinking water three days prior to and throughout the experimental protocol. Animals were subjected to either bilateral (study-1) or unilateral (study-2) injections of CTX (25µl at 10µM) into the TA muscle and tissues were harvested 3, 10 and 21 days post injection (study-1)

or 4, 7, 14 and 21 days post injection (study-2). Also, for reference of normal skeletal muscle architecture, a non-injured, non-supplemented group (n=8) was included (study-1). All procedures were conducted according to guidelines established by the Canadian Council on Animal Care with ethics approval from the McMaster University Research Ethics Board.

*Histology.* Muscles were formalin-fixed, paraffin-embedded and stained with hematoxylin and eosin (H&E) to reveal skeletal muscle architecture and viewed on a Nikon Eclipse 90i and mean muscle fibre CSA was calculated by analyzing 300 fibres per animal from randomly captured images in a blind fashion using Nikon NIS Elements 3.0 software. β-gal activity in Myf5-LacZ cells was visualized as previously described [18]. Briefly, cells were fixed in 2 mM MgCl2 and 0.25% glutaraldehyde in PBS for 10 min followed by washing and overnight incubation in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 4 mg/ml X-gal and 0.02% NP40 in PBS at 37°C. Cells were then washed in PBS, fixed for 10 min with 4% PFA and mounted

*Cell culture.* All cultures were maintained at 37°C in 5% $CO_2$. Primary myoblasts were isolated from wild-type C57Bl/6 and AT1a$^{-/-}$ mice as previously described [19] and cultured in primary growth media (PGM, 20% FBS in Hams F10 with 2.5 ng/mL bFGF and antibiotics). Myf5-LacZ myoblasts were a generous gift from Dr. Michael Rudnicki (Ottawa Hospital Research Institute, Ottawa Canada) and were cultured in PGM. C2C12 myoblasts were cultured in growth medium (GM, DMEM supplemented with 10% fetal bovine serum and antibiotics) or serum free

medium (SFM, DMEM supplemented with antibiotics). When indicated, C2C12 and Myf5-LacZ myoblasts were rendered quiescent through 72h of culturing in a methocellulose supplemented medium that prevented cell adhesion as described previously [20].

*Flow cytometery.* The effect of Ang II treatment (24h, 10μM) on cell cycle kinetics of methocellulose cultured C2C12 cells was determined using PI staining or a commercially available BrdU/7AAD kit as per the manufactures instructions (cat#559619, BD pharmagen, USA) and analyzed using flow cytometry (Epics Altra, Beckman Coulter, USA).

*RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction.* RNA was isolated from quiescent C2C12 myoblasts (with and without Ang II (10μM) treatment for 3h, 6h or 12h) using the RNeasy method according to the manufactures instructions (Qiagen Sciences, USA) and analyzed using quantitative RT-PCR (qRT-PCR). Gene expression fold change was calculated using the delta-delta Ct method [21] using ribosomal protein L32 as a housekeeping gene. Primer sequences can be found in Table 1.

*Transwell/invasion/checkerboard assays.* Transwell assays were conducted using proliferating or quiescent C2C12 myoblasts and primary or $AT1a^{-/-}$ myoblasts as described previously [22] with minor modifications using 6 or 24 well, 8μm pore transwell systems. Cells were allowed to migrate for 12h with the lower chamber of the well containing GM, 20% FBS in DMEM, PGM or SFM with or without Ang II (10μM) or an MMP2 inhibitor (1μM; cat# 44424, Calbiochem,

USA) where indicated. Invasion and checkerboard assays were performed in the same fashion as migration assays with the exception that transwells were pre-coated with 1% gelatin or media on either the top or the bottom of the transwell was supplemented with Ang II (10μM). Migration was assessed by staining the cells with crystal violet, removing the cells on the upper side of the transwell and counting the number of migrated cells in 15 random fields of view at 40x magnification using Zeiss Axiovert 200 microscope (Carl Zeiss Canada Ltd.) or by solubilizing the cells in 1% triton X-100 and measuring the absorbance of the triton X-100 solution using an Ultraspec 3000 Pro (GE Healthcare, USA) at 595nm.

*Under agarose (UA) migration assay.* To further assess the chemotactic capacity of C2C12 cells in response to Ang II treatment, an under agarose migration assay [23] was performed as depicted in Figure S3. 1% agarose was polymerized in 10% FBS in DMEM in 35 mm culture plates and 3 wells were cut. C2C12 cells were added to the centre well while GM with or without Ang II (100μM) was added to the outer wells and allowed to incubate for 14h. The total number of cells and distance migrated under the agarose was analyzed using a Zeiss Axiovert 200 microscope (Carl Zeiss Canada Ltd). Maximal migration distance was calculated by averaging the distance of the top 15 migrating cells per sample.

*Immunohistochemistry.* C2C12 cells were plated into the centre well of an under agarose migration plate while GM with or without Ang II (100μM) was then added

to one of the outer wells and incubated for 6h. Cells were fixed in 4% PFA for 10 min (or 5 min, followed by dehydration in ethanol for co-staining). IHC staining for AT1 receptor was done using anti-AT1 (cat# SC-1173, Santa Cruz, USA) and revealed with anti-rabbit Alexa 488 secondary antibody (Molecular Probes, USA). Filamentous actin was visualized with TRITC-conjugated phalloidin (0.2µg/mL, cat#P1951, Sigma-Aldrich, Canada) and incubated simultaneously overnight at 4°C with anti-AT1 for costaining and counterstained with DAPI.

*Mechanical stretch/coculture migration assay.* Flexible bottom culture plates (Flexcell International, USA) were modified to accommodate a transwell insert. C2C12 myoblasts were pretreated for 36h in either GM or GM supplemented with captopril (10mM) to inhibit endogenous Ang II production. $2 \times 10^5$ cells were seeded onto type I collagen coated flex-cell plates while $5 \times 10^4$ cells were seeded into the upper well of a transwell insert and placed into the flex-cell plates. Cells were subjected to a 4h cyclic strain protocol (of 0.1 Hz at 20% strain) applied using the FX-4000 Tension Plus (Flexcell International, USA) in the presence or absence of captopril. After 20h, cell migration was assessed as described above.

*Gelatin Zymography.* C2C12 myoblasts were cultured in GM and treated with Ang II (10µM) for 48h and media and total protein was collected analyzed using gelatin zymography as previously described [24]. 20µl of media or 7.5µg of protein was loaded into a 10% polyacrylamide gel containing 1% gelatin and resolved for 90 min at 100 V. Gels were incubated in 2.5% triton X-100 for 1h,

washed in $H_2O$ for 2 x 20min, and incubated for 20h at 37 °C in a 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM calcium chloride. Gels were then fixed with 50% methanol, 10% acetic acid containing 0.25% Coomassie Blue. Bands appear clear against the stained gel and were visualized using an Alpha Innotech FluroChem SP and quantified using Alphaease software.

*Statistical analysis.* Analysis of *in vivo* measures was done by two-way ANOVA. In vitro experiments were analyzed using t-tests and one-way ANOVA where appropriate. T-tests were also performed on gene expression fold change for qRT-PCR analysis.

**Results**

*Ang II signaling mediates skeletal muscle growth following CTX injury.* To determine the *in vivo* consequence of impaired Ang II signaling on muscle regeneration and growth we injected $AT1a^{-/-}$ or ACE inhibitor (captopril) treated mice with CTX to induce myotrauma. Analysis of H&E stained TA cross-sections revealed that in comparison to controls, captopril supplemented mice presented with a decrease in muscle fibre cross-sectional area (CSA) of ~25% 21 days following injury (Figure. 1A, B, C; $p < 0.05$) while no differences were observed between groups 10 days post injection. 21 days also appeared to be sufficient time for control animals to fully regenerate as fibre CSA was not different from uninjured (UI) mice while captopril treated mice still demonstrated a significant ~25% reduction in CSA relative to UI mice (Figure 1C). Analysis of small

(<1500μm), medium (1500 to 3000μm) and large (>3000μm) size fibres revealed an increased frequency of small fibres and a decrease in large fibres with captopril treatment (Figure 1D). Furthermore, when muscle fibre CSA was analyzed from days 10-21 post injury we observed that captopril treatment significantly impaired muscle growth as control animals demonstrated an 81% increase in CSA while captopril treated mice increased by only 27% (Figure 1C). To investigate the role of Ang II receptor sub-type we repeated similar experiments inducing regeneration in $AT1a^{-/-}$ mice. In agreement with the captopril treated animals, $AT1a^{-/-}$ mice also revealed impaired skeletal muscle growth following the formation of myofibres with significant decreases in fibre CSA of ~35% and ~25% at 14d and 21d respectively compared to controls (Figure 1E). When the rate of growth was determined between day 7-14 post injury, a 110% increase in fiber CSA was documented in the WT mice while a non-significant 19% increase was observed in $AT1a^{-/-}$ mice (Figure 1E). Importantly, normal skeletal muscle growth was not impaired in $AT1a^{-/-}$ mice as no differences were observed in myofibre CSA between uninjured WT and $AT1a^{-/-}$ mice (data not shown), however our data suggests that post-natal induced muscle growth following injury requires Ang II.

*Ang II activates quiescent myoblasts without enhancing proliferation.* To investigate the mechanisms responsible for repressed muscle growth, due to inhibition of Ang II signaling, we analyzed the effect of Ang II treatment on myoblast function. The use of methocellulose culture suspension to induce

quiescence has previously been validated and is a valuable tool to assess myoblast activation [25-27]. To confirm quiescence of methocellulose treated cells we analyzed the cell cycle kinetics using propidium iodide staining and flow cytometry following 72h of methocellulose treatment. When compared to actively dividing C2C12 myoblasts, 72h of methocellulose suspension induced synchronization of cells in G1, reduced the number of actively dividing cells (S-phase cells) to less than 1.5% (Figure S1) and decreased the number of cells progressing through the cell cycle (28% in controls vs. 11% in methocellulose treated, (Figure S1)). To assess if Ang II could activate myoblasts, quiescent C2C12 cells were treated with Ang II for 3, 6 or 12h. Treatment of quiescent cells with Ang II induced the upregulation of mRNA species characteristic of activated myoblasts such as increases in the myogenic regulatory factor Myf5 (2.5-fold) and MyoD (6-fold) at 3h and 6h respectively as well as Pax7 (7-fold, 6h) (Figure 2A, B, C). Ang II treatment also led to a significant increase in the cell cycle gene cyclin D1 as well as an early (3h) increase in cfos gene expression (Figure 2D, E), which has been proposed to be one of the earliest events associated with satellite cell activation *in vivo* [3]. Additionally, Ang II treatment increased the expression of the AT1 receptor in quiescent myoblasts as a 5-fold increase was observed following 6h of treatment (Figure 2F). Since satellite cell activation is characterized by an upregulation of Myf5 [5] and to confirm that Ang II activated quiescent primary myoblasts, the effect of 6h of Ang II treatment was assessed in Myf5-LacZ primary myoblasts following 72h of methocellulose culture

suspension. In comparison to actively dividing cells, methocellolose culture reduced the number of β-galactosidase (β-gal)+ cells from ~77% to ~21% (Figure 3A; $p<0.05$) confirming the quiescence of these cells. However, Ang II treatment of quiescent myoblasts successfully increased the number of β-gal+ cells by ~75% (Figure 3A; $p<0.05$).

Next, we wanted to assess whether the activation of quiescent myoblasts induced cell motility. Therefore, we analyzed the migratory capacity of quiescent myoblasts treated with serum free media (methocel), Ang II or 20% FBS supplemented media (control), which is a known inducer of myoblast activation. When compared to controls, cells treated with SFM displayed a severe impairment of migratory capacity (~70% reduction, Figure 3B) confirming that quiescent cells must become activated in order to undergo migration. Interestingly, when comparing the migratory activity of cells treated with SFM or Ang II, we demonstrate that both direct treatment and a concentration gradient of Ang II significantly increased the chemokinetic migration of cells by ~75% (Figure 3B) demonstrating a functional measure of myoblast activation.

Since we demonstrated that Ang II activates quiescent cells and upregulates key transcription factors necessary for myoblast proliferation (i.e. Myf5, cyclin D1), we tested whether Ang II possessed the ability to induce quiescent cells to proliferate. Interestingly, no differences were observed between groups in either the number of BrdU positive cells or the cell cycle kinetics in response to 24h of Ang II treatment of quiescent myoblasts (Figure

S2). Collectively, these results demonstrate that Ang II activates myoblasts and initiates cell cycle entry but does not directly induce proliferation of quiescent myoblasts.

*Ang II signals through the AT1a receptor to induce chemotaxis of proliferating cells.* Since the process of myoblast chemotaxis is poorly understood and Ang II has been implicated in the motility of several cell types [12-14], we assessed the role of Ang II in regulating myoblast chemotaxis using an assay commonly utilized to assess inflammatory cell migration [23]. Under agarose migration analysis (Figure S3) revealed a robust 133% increase in the number of C2C12 cells that migrated out of the centre well in response to an Ang II concentration gradient (Figure 4C) as well as increasing the maximal distance migrated by ~50% (Ang II-298.3μm vs. Con-201.7μm $p<0.05$; Figure 4D). This increase in C2C12 migration was also evident using transwell assays where Ang II treatment induced a 30% increase in migratory capacity (Figure 4B). There are two distinct forms of myoblast motility: 1. chemokinesis, analogous to stochastic movements, and 2. chemotaxis, analogous to directed homing. Checkerboard assays revealed that when activated myoblasts were treated directly with Ang II no increase in migration was observed; however, when exposed to a concentration gradient of Ang II, a significant increase in the number of migrating cells was demonstrated (Figure S4A). To confirm that Ang II augments primary myoblast migration and to delineate which receptor subtype was activated during Ang II-induced myoblast chemotaxis, primary myoblasts were harvested from

C57Bl/6 (wild-type; WT) and AT1a$^{-/-}$ mice. Transwell assays revealed that exogenous Ang II treatment significantly increased primary myoblast chemotaxis by 43% while AT1a$^{-/-}$ myoblasts demonstrated a profound (~62%) inhibition ($p<0.05$) of migratory capacity and did not respond to Ang II treatment (Figure 4A).

We have previously demonstrated that myoblasts locally produce Ang II and express a "stretch-responsive" local angiotensin signaling system [15]. Based on these findings, we explored the possibility that the mechanical stretch of myoblasts could induce the production of Ang II, which in turn, could initiate the migration of cocultured myoblasts. Our results demonstrate a significant 17% increase in myoblast migration in response to factors released from myoblasts undergoing stretch (Figure 5A) that was abolished when myoblasts underwent mechanical stretch in the presence of captopril (Figure 5B). Collectively, these data indicate that Ang II signaling through the AT1a receptor is a mediator of myoblast chemotaxis and that Ang II can signal chemotaxis in a paracrine fashion.

*Ang II-induced chemotaxis is mediated by multiple mechanisms including cytoskeletal reorganization and increased MMP2.* Since proper actin filament assembly has been hypothesized to be a prerequisite for directed cellular motility [28], and implicated in Ang II-induced migration of other cell types [29, 30], we treated C2C12 myoblasts with a concentration gradient of Ang II and assessed the number of cells displaying altered cytoskeletal characteristics. TRITC-

conjugated phalloidin staining of the actin cytoskeleton revealed that Ang II treatment increased the number of cells displaying lamellipodial projections by 111% (Figure 6D-F). Interestingly, IHC costaining of AT1 and phalloidin revealed that AT1 translocates to the leading edge of the cell and was concentrated in lamellipodia (Figure S5). These results indicate that Ang II signaling induced cellular polarization and reorganization of the actin cytoskeleton. Another important component of cell migration is the ability of a cell to degrade its extracellular environment to promote cell motility. Therefore, we assessed whether Ang II influenced enzyme activity involved in the breakdown of the extracellular matrix (ECM). Gelatin zymography analysis demonstrated an ~40% increase in total MMP2 activity in both the cell culture media and cell lysate (Figure 6A, B) of C2C12 cells treated with Ang II. These results are in agreement with *in vitro* invasion assays, which demonstrated that Ang II treatment of myoblasts induced a significant increase in the number of cells invading the gelatin coated transwells (Figure S4B), presumably by inducing the enzymes involved in the degradation of extracellular matrix proteins. To confirm the importance of Ang II-induced MMP2 activity in myoblast migration, a transwell assay was conducted in the presence of an MMP2 inhibitor (MMP2I). Results revealed that inhibition of MMP2 did not appear to affect basal migration of C2C12 cells, however, when cells were treated with Ang II and the MMP2I, the observed increase in migratory capacity was completely abolished (Figure 6C).

These data suggest that Ang II-stimulated chemotaxis was regulated, at least in part, by MMP2 activity.

**Discussion**

The regeneration and growth of skeletal muscle is largely dependent on the capacity of muscle satellite cells to efficiently respond to directed cues that signal the activation, motility and progression of satellite cells through the myogenic program. An impairment of any of these processes could result in the decreased capacity for repair or growth. Consequently, we have identified Ang II as a novel regulator of muscle stem cell chemotaxis, an activator of quiescent myoblasts and a necessary factor for induced myofibre growth following injury.

In recent years, Ang II has been described as having wide ranging biological effects independent of its vasoactivity [31]. The function of Ang II signaling in skeletal muscle and associated muscle satellite cells remains incompletely described with the potential to influence muscle hypertrophy [16, 17]. Our *in vivo* observations support a regulatory role for Ang II in mediating muscle fibre growth likely through muscle satellite cell activation and chemotaxis. Specifically, we demonstrate that both captopril treatment and ablation of the AT1a receptor resulted in a similar inhibition of fibre growth highlighting a role for AT1 mediated signaling. Importantly, muscle fibre CSA was not different between uninjured control and $AT1a^{-/-}$ mice. This indicates that Ang II likely does

not influence embryonic or neonatal muscle development but appears to only influence postnatal myogenesis during regeneration and growth.

To explain the observed inhibition of skeletal muscle growth due to captopril treatment, we focused our analysis on the potential of Ang II to mediate the early response of muscle stem cells to muscle repair since very little is known regarding the factors that mediate this phase. We demonstrate that Ang II induced the expression of mRNAs and protein known to be upregulated in activated myoblasts such as the myogenic regulatory factors Myf5, MyoD and Pax7. Interestingly, the temporal expression of these genes in response to Ang II treatment is in agreement with *in vivo* data demonstrating that satellite cell activation is represented by an early upregulation of Myf5 followed by increased MyoD expression [32]. Importantly, Ang II treatment lead to the production of functional Myf5 protein in cultured primary cells proving that effects of Ang II on myoblast activation are not restricted to C2C12 cells. We also demonstrated that the activation of myoblasts resulted in acquired cellular motility, which likely serves to upregulate the migratory machinery necessary to respond to chemotactic signals. It is interesting to note that Ang II treatment of quiescent cells induced the same magnitude increase in chemokinesis of C2C12 cells as that observed in the number of β-gal+ Myf5-LacZ cells, further supporting the relationship between Ang II induced activation and cellular motility. These results suggest that early responses of satellite cells to myotrauma may be coordinated

by Ang II as it plays a pleiotropic role in activating cells as well directing their subsequent chemotactic response.

Our data also demonstrate that although Ang II activated myoblasts, it did not alter cell cycle kinetics of quiescent cells. These data suggest that Ang II primarily functions to activate myoblasts but may act in concert with other factors to induce full cell cycle entry and proliferation. This theory is supported by Hlaing and colleagues [33] who demonstrated that Ang II treatment of serum starved C2C12 cells had no effect on myoblast proliferation but induced the transient activation of Cdk4, Rb phosphorylation and the subsequent release of HDAC1. However, they also reported that although Ang II transcriptionally activated E2F-1, this complex did not dissociate from the Rb protein subsequently suppressing genes necessary for cell cycle progression.

The chemotaxis of muscle stem cells to the site of injury is an ill defined, necessary component of the early response of satellite cells to myotrauma. Using several techniques, the present experiments demonstrate that exogenous Ang II treatment significantly increased both primary and C2C12 chemotaxis. We have previously reported that myoblasts have the ability to secrete Ang II and that mechanical stimulation of C2C12 myoblasts resulted in the upregulation of RAS family member gene expression [15]. Here, we demonstrate that stretch-induced release of Ang II at "physiological" levels results in paracrine signaling to significantly increase myoblast motility. Although the magnitude of increase was less than that induced by Ang II treatment, the concentration of Ang II released

into the culture media was likely magnitudes lower, highlighting the robustness of the effect of Ang II on motility. These results suggest that mechanical stimulation due to muscle contraction *in vivo* may stimulate Ang II release that functions to recruit muscle satellite cells to the site of injury. Ang II treatment of both quiescent and proliferating cells resulted in enhanced cellular motility with quiescent cells responding to Ang II treatment chemokinetically and proliferating cells undergoing chemotaxis. Based on our results, we propose that Ang II functions to activate quiescent myoblasts subsequently increasing their motility but then serves to direct the homing response following their activation.

Chemotaxis can be best viewed as a cyclical process involving cellular polarization, extension, contraction and detachment [28, 34, 35]. Interestingly, Ang II appeared to enhance numerous aspects of this process. Integral to the initiation of chemotaxis is the formation of actin-rich lamellipodia that serve to directionally extend the migrating cell forward [36]. The present results demonstrate that Ang II treatment induced cytoskeletal reorganization and increased the number of cells possessing lamellipodia consistent with other reports demonstrating actin cytoskeletal rearrangement and lamellipodial formation in Ang II induced migration [29, 30]. MMP2 is a gelatinase that is known to regulate migration by facilitating detachment from the ECM as well as increasing the space for cellular expansion as the cell migrates toward the degraded matrix [34]. We chose to focus our attention on MMP2 for two reasons. Firstly, this is the primary MMP expressed in myoblasts [37] and secondly, we

demonstrate that Ang II increased myoblast invasion through gelatin covered transwells. In agreement with the current study, El Fahime and colleagues [37] reported that pharmacological inhibition of MMPs severely inhibited *in vivo* migration of transplanted C2C12 myoblasts while overexpression of MMP2 significantly increased their *in vivo* migratory capacity.

Ang II possesses the capacity to bind to two distinct receptor subtypes, AT1 and AT2 [38]. We have previously demonstrated that both C2C12 and primary myoblasts express both of these receptor subtypes and therefore either could regulate Ang II mediated migration. Our results highlight the role of AT1a signaling as $AT1a^{-/-}$ primary myoblasts were severely impaired (62% reduction compared to controls) in their basal migratory capacity and did not respond to exogenous Ang II treatment. Interestingly, IHC staining of the AT1 receptor revealed its localization to lamellipodial projections in myoblasts. The functional significance of this relationship is currently unknown, however, the G-coupled chemokine receptors CCR2 and CCR5 redistribute to the leading edge of the cell during chemotaxis in lymphocytes (Nieto et al. 1997) and natural killer cells [39]. Similarly, the receptor for urokinase-type plasminogen activator undergoes translocalization to the leading edge of migrating human monocytes [40]. Therefore, the localization of AT1 at the leading edge may promote site directed accumulation of pro-migratory signalling molecules.

Collectively, the findings presented in this manuscript identify Ang II as a novel regulator of skeletal muscle growth through modulating muscle satellite cell

activation and migration. Clinically, ACE inhibitors and angiotensin receptor blockers are amongst the most commonly prescribed medications [9] with >65 million individuals in the United States considered clinically hypertensive with the highest prevalence in elderly [41, 42]. Unfortunately this population is also at the highest risk of muscle loss due to age. This punctuates the importance of understanding the role of Ang II in skeletal muscle and highlights the need for further investigation into the effects of Ang II signaling inhibition in elderly individuals undergoing pharmacological blockade of Ang II signaling.

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**Figure legends.**

**Figure 1. Inhibition of Ang II signaling abrogates skeletal muscle growth following CTX injury.** Representative H&E stains of TA cross-sections of (A) control and (B) captopril treated mice 21 days after CTX injection (both photos - 40x magnification). C) Mean muscle fibre CSA of control and captopril treated mice 10 and 21 days following CTX injection. Note: ◊ - indicates CSA of uninjured mice (n=6 per group). D) Distribution of small (<1500μm$^2$), medium (1500-3000μm$^2$) and large (>3000μm$^2$) size muscle fibres of control and captopril treated mice 21 days following CTX injection. E) Analysis of muscle fibre CSA of control and AT1a$^{-/-}$ mice 7, 14 and 21 days following CTX injection (n=4 per group). Data are presented as mean ± s.e.m, *indicates a significant difference ($p<0.05$) from control.

**Figure 2. Ang II treatment upregulates mRNAs found within activated satellite cells.** qRT-PCR analysis of (A) Myf5, (B) MyoD, (C) Pax7, (D) cfos, (E) cyclin D1, and (F) AT1 in quiescent C2C12 myoblasts in response to Ang II treatment for 3, 6 or 12h (n=6 per group). Data are presented as mean ± s.e.m, *indicates a significant difference ($p<0.05$) from control.

**Figure 3. Ang II treatment results in the activation of primary myoblasts and the acquisition of motility.** A) Actively dividing Myf5-LacZ myoblasts (control) were cultured in PGM while quiescent Myf5-LacZ myoblasts were

treated with Hams F10 (methocel) or Ang II for 6h and the percentage of β-gal+ cells was assessed (n=6 per group). B) Quiescent C2C12 myoblasts were treated with SFM (methocel), Ang II directly (Ang II treatment), an Ang II concentration gradient (Ang II on bottom) or 20% FBS (control) and migration was measured using transwells (n=6 per group). Data are presented as mean ± s.e.m, *indicates a significant difference ($p<0.05$) from control.

**Figure 4. Ang II signals through the AT1a receptor to increase myoblast chemotaxis.** Analysis of (A) primary WT, AT1a$^{-/-}$ and (B) C2C12 myoblast migration through transwells in response to Ang II treatment (n=10 per group). Analysis of (C) total number and (D) maximal distance of C2C12 myoblasts following 12h of UA migration (n=16 per group). *indicates a significant difference ($p<0.05$) from control.

**Figure 5. Mechanical stretch stimulates Ang II secretion and signals and induces chemotaxis in coculture.** Analysis of C2C12 chemotaxis in response to coculture with myoblasts exposed to 4h of cyclic mechanical stretch in the (A) absence or (B) presence of captopril (n=6 per group). *indicates a significant difference ($p<0.05$) from control-unflexed. All data are presented as mean ± s.e.m.

**Figure 6. Ang II treatment regulates myoblast chemotaxis through increased MMP2 and actin cytoskeletal reorganization.** A) Gelatin zymography analysis of total MMP2 activity within cell culture media and (B) cell lysates of C2C12 myoblasts treated with Ang II (n=6 per group). C) Analysis of the migration of C2C12 myoblasts treated with Vehicle, Vehicle+Ang II, MMP2 inhibitor or Ang II+MMP2 inhibitor (n=6 per group). F) Analysis of TRITC-conjugated phalloidin staining of (D) control and (E) Ang II treated C2C12 myoblasts (20x magnification, n=12 per group). Note: arrows indicate cells displaying lamellipodial projections. Data are presented as mean ± s.e.m, *indicates a significant difference ($p<0.05$) from control.

**Supplemental figure legends.**

**Figure S1. Methocellulose culturing synchronizes C2C12 cells in G1.** Representative cell cycle profile of Flow analysis of PI stained C2C12 cells cultured in (A) GM or (B) 1.5% methocellulose for 72h (n=5 per group).

**Figure S2. Ang II treatment does not induce proliferation or alter cell cycle kinetics of C2C12 myoblasts.** Representative flow cytometery profiles of BrdU staining of (A) quiescent control and (B) Ang II treated C2C12 cells (n=6 per group). Representative cell cycle profiles of 7AAD staining in (C) control and (D) Ang II treated C2C12 cells (n=6 per group).

**Figure S3. Depiction of the under agarose migration assay.**

**Figure S4. Ang II treatment increases myoblast chemotaxis and invasion.** A) Analysis C2C12 myoblasts either directly treated (Ang II on top) or subjected to a concentration gradient (Ang II on bottom) of Ang II. B) Analysis of control and Ang II treated C2C12 cells to invade gelatin coated transwells (n=6 per group). Data are presented as mean ± s.e.m *indicates a significant difference ($p<0.05$) from control.

**Figure S5. AT1 colocalizes with lamellipodial projections.** IHC staining of (A) DAPI, (B) AT1, (C) phalloidin and (D) merge in C2C12 myoblasts (100x

magnification). Arrows indicate colocalization of AT1 to lamellipodial projections of a polarized cell.

**Tables.**

**Table 1. Primer sequences used.**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| AT1 | ACAGTGATATTGGTGTTCTCAATGAAA | CCATTGTCCACCCGATGAA |
| cfos | GAATGGTGAAGACCGTGTCA | TGCAACGCAGACTTCTCATC |
| Cyclin D1 | TGAACTACCTGGACCGCTTC | CCACTTGAGCTTGTTCACCA |
| Myf5 | TGAAGGATGGACATGACGGACG | TTGTGTGCTCCGAAGGCTGCT |
| MyoD | TACCCAAGGTGGAGATCCTG | CATCATGCCATCAGAGCAGT |
| Pax7 | CTGGATGAGGGCTCAGATGT | GGTTAGCTCCTGCCTGCTTA |
| L32 | TCCACAATGTCAAGGAGCTG | ACTCATTTTCTTCGCTGCGT |

**Figures.**

**Figure 1.**

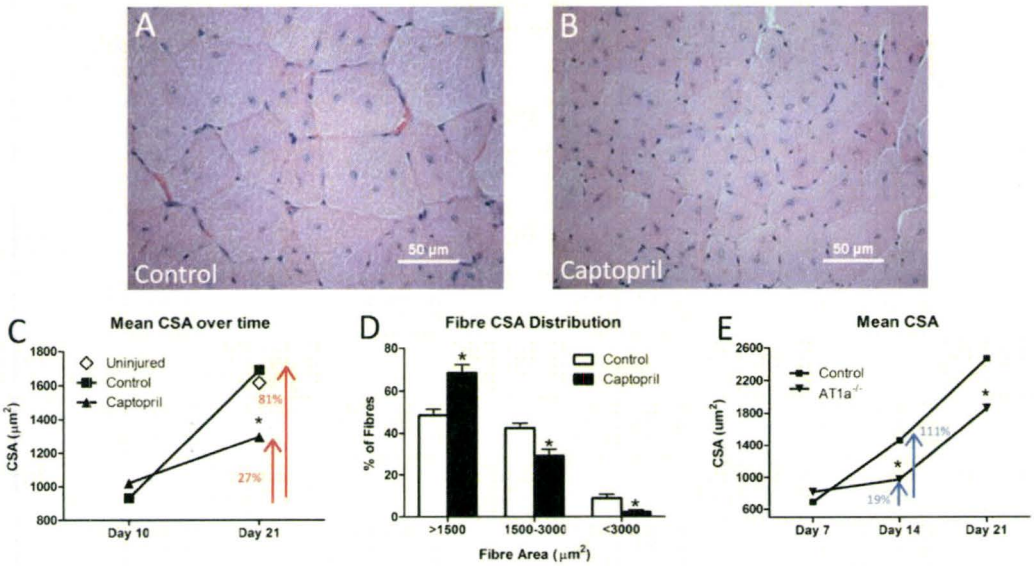


**Figure 2.**

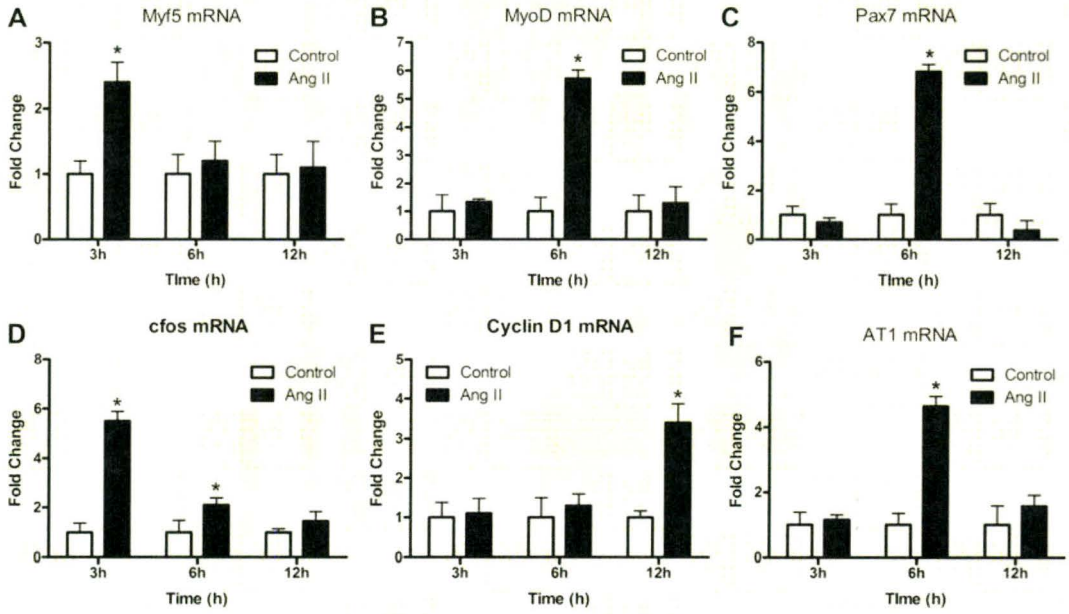

A
Myf5 mRNA
Control
Ang II
Fold Change
Time (h)
3h
6h
12h
B
MyoD mRNA
C
Pax7 mRNA
D
cfos mRNA
E
Cyclin D1 mRNA
F
AT1 mRNA

**Figure 3.**

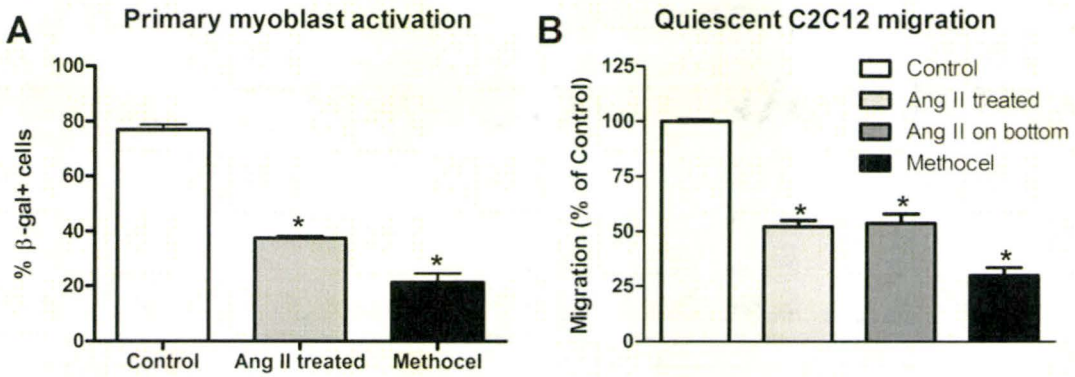

A
Primary myoblast activation
% β-gal+ cells
100
80
60
40
20
0
*
*
Control
Ang II treated
Methocel
B
Quiescent C2C12 migration
Migration (% of Control)
125
100
75
50
25
0
Control
Ang II treated
Ang II on bottom
Methocel
*
*
*

**Figure 4.**

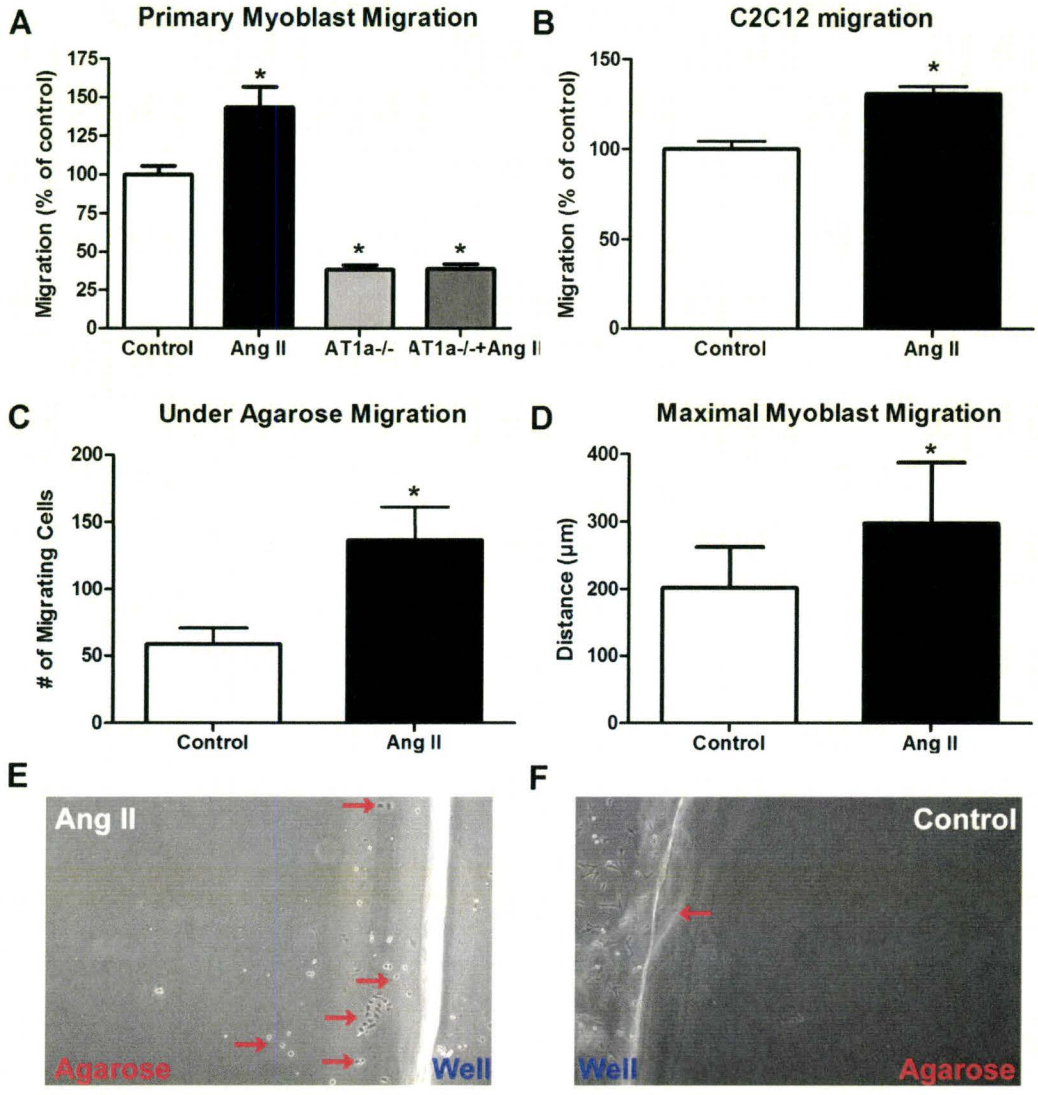

A
Primary Myoblast Migration
Migration (% of control)
175
150
125
100
75
50
25
0
*
*
*
Control
Ang II
AT1a-/-
AT1a-/-+Ang II
B
C2C12 migration
Migration (% of control)
150
100
50
0
*
Control
Ang II
C
Under Agarose Migration
# of Migrating Cells
200
150
100
50
0
*
Control
Ang II
D
Maximal Myoblast Migration
Distance (µm)
400
300
200
100
0
*
Control
Ang II
E
Ang II
Agarose
Well
F
Control
Well
Agarose

**Figure 5.**

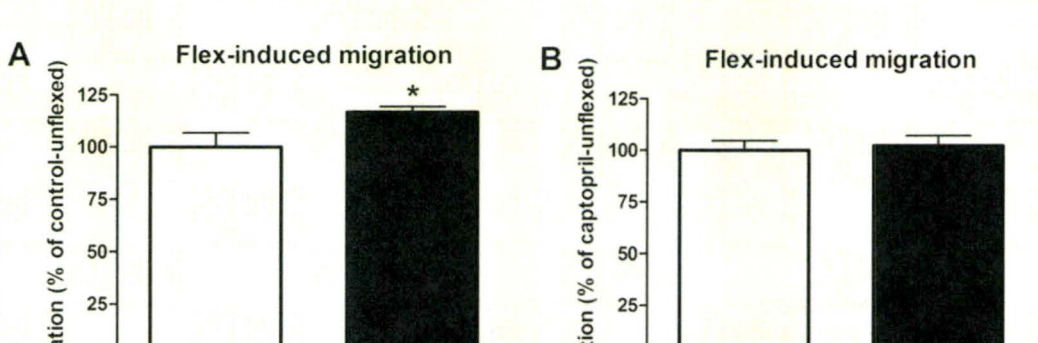
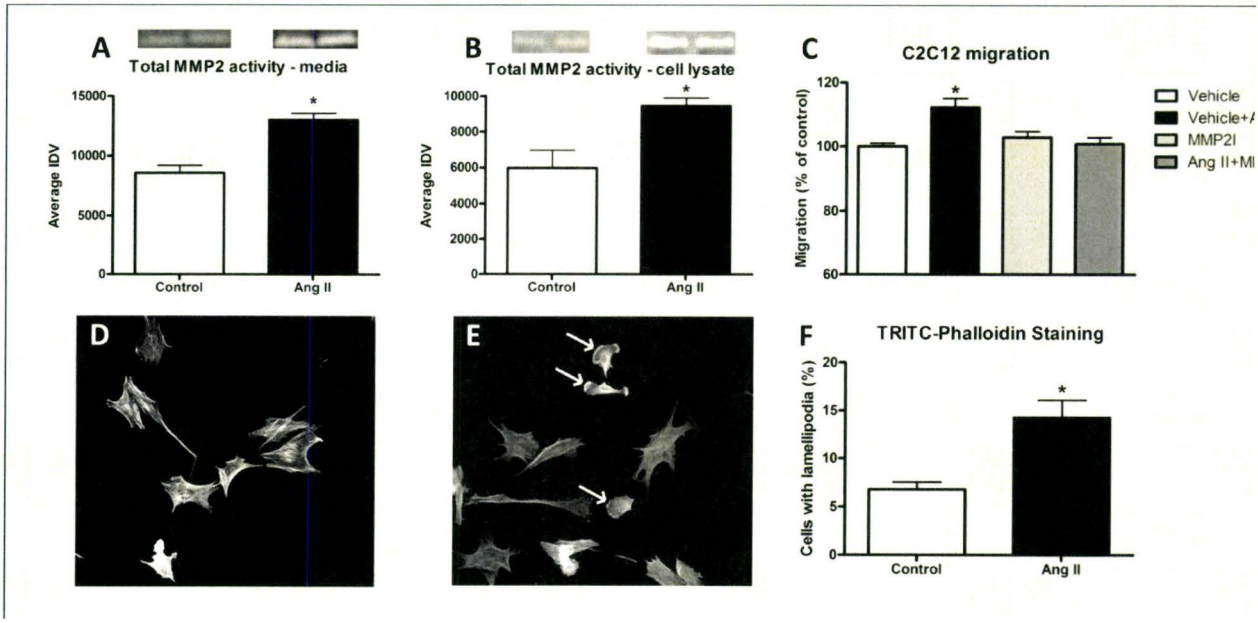

A
Flex-induced migration
Migration (% of control-unflexed)
125
100
75
50
25
0
*
Control-unflexed
Control-flexed
B
Flex-induced migration
Migration (% of captopril-unflexed)
125
100
75
50
25
0
Captopril-unflexed
Captopril-flexed

**Figure 6.**


A
Total MMP2 activity - media
Average IDV
15000
10000
5000
0
Control
Ang II
B
Total MMP2 activity - cell lysate
Average IDV
10000
8000
6000
4000
2000
0
Control
Ang II
C
C2C12 migration
Migration (% of control)
120
100
80
60
Vehicle
Vehicle+A
MMP2I
Ang II+MI
D
E
F
TRITC-Phalloidin Staining
Cells with lamellipodia (%)
20
15
10
5
0
Control
Ang II

**Supplemental figures.**

**Figure S1.**

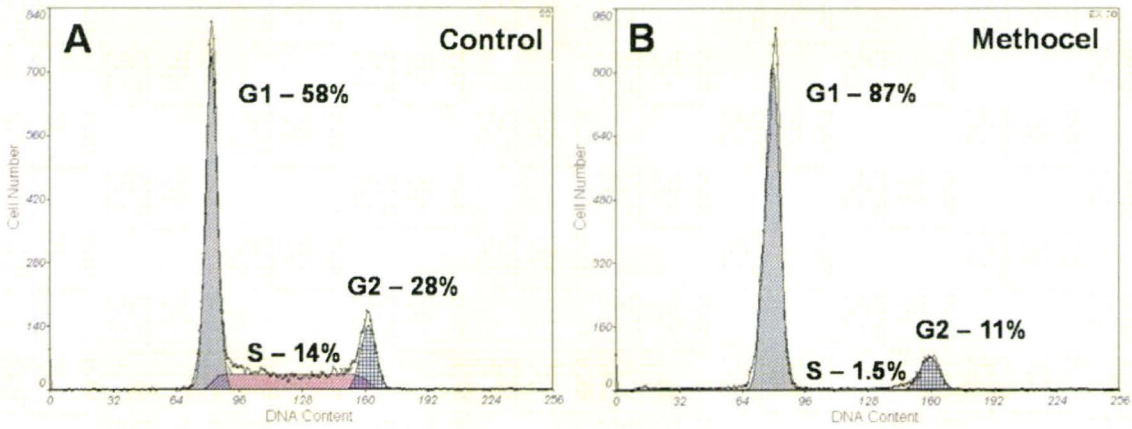


**Figure S2.**

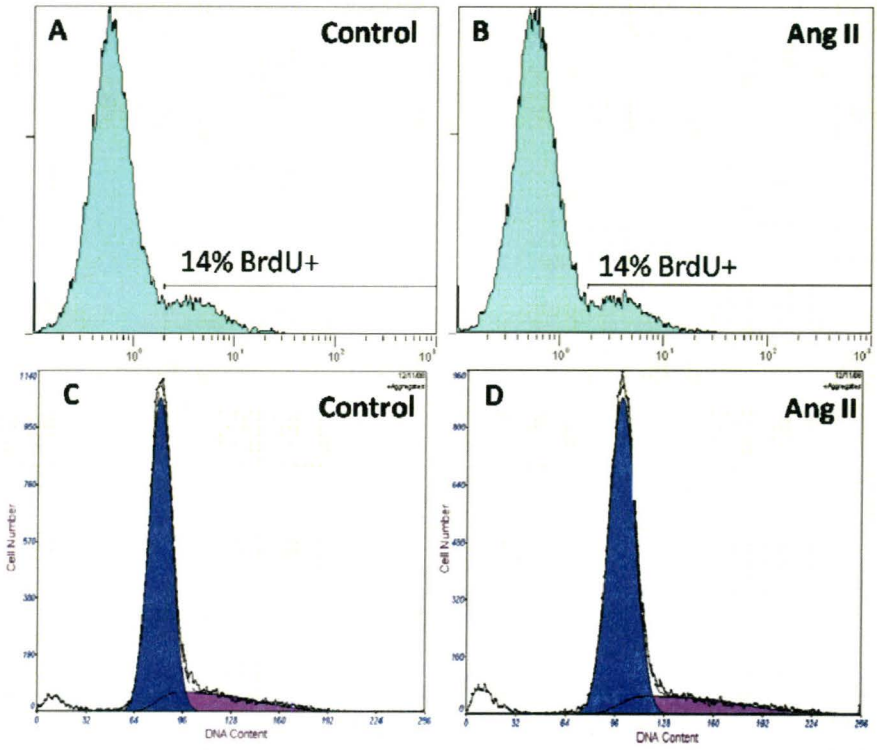

A
Control
14% BrdU+
B
Ang II
14% BrdU+
C
Control
Cell Number
DNA Content
D
Ang II
Cell Number
DNA Content

**Figure S3.**

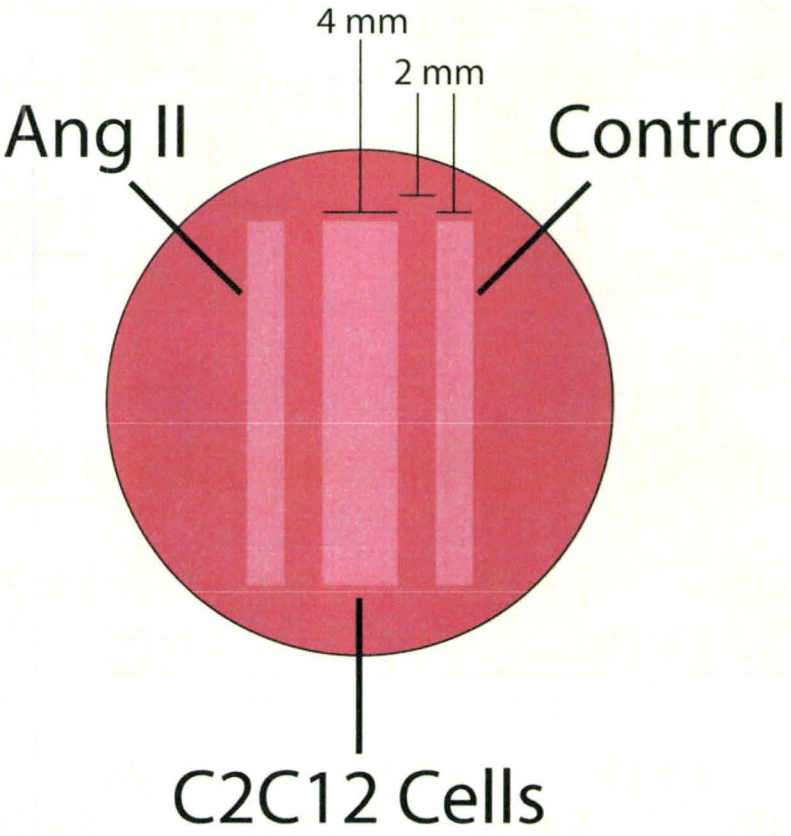


**Figure S4.**

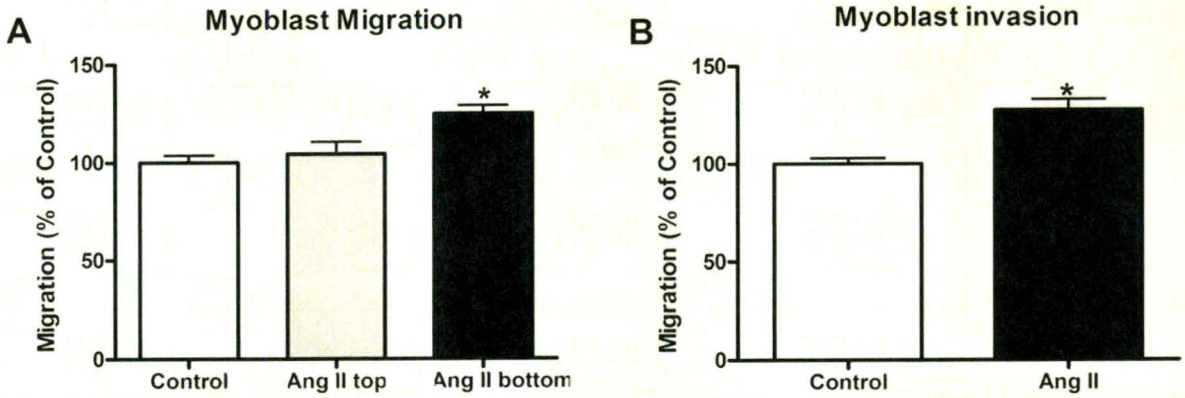
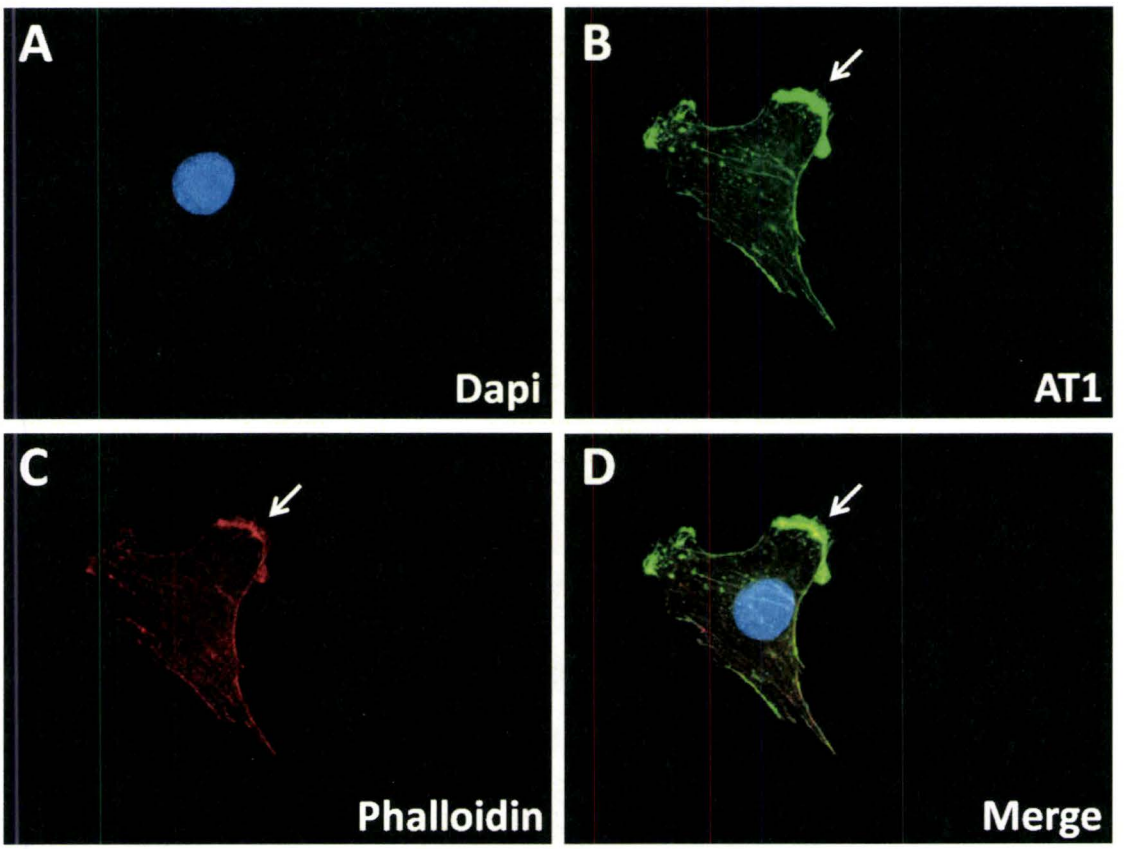

A
Myoblast Migration
Migration (% of Control)
150
100
50
0
*
Control
Ang II top
Ang II bottom
B
Myoblast invasion
Migration (% of Control)
150
100
50
0
*
Control
Ang II

**Figure S5.**



## Chapter 4: Manuscript 3

# Captopril treatment induces hyperplasia but inhibits myonuclear accretion following severe myotrauma

***American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.*** **In Review.**

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**Running head**: Angiotensin II regulates myonuclear addition

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**Key Words:** Satellite cell, Angiotensin II, muscle growth, differentiation, myonuclear accretion, renin-angiotensin system

**Abstract**

The role of Ang II in skeletal muscle and muscle satellite cells is largely unknown. To investigate whether muscle injury activates a local angiotensin II (Ang II) signaling system in skeletal muscle we injected cardiotoxin (CTX) to induce muscle regeneration. IHC analysis revealed a robust increase in angiotensinogen and the angiotensin type 1 (AT1) receptor staining intensity during myogenesis. Interestingly, AT1 was downregulated as regeneration progressed. To assess the contribution of this local system to skeletal muscle regeneration, nuclear accretion and fibre formation we also induced muscle injury in mice supplemented with captopril (angiotensin converting enzyme (ACE) inhibitor). We demonstrate that captopril treatment significantly inhibited the accretion of nuclei in myofibres by ~25% while increasing the total number of fibres in the TA by 37%. These effects appear to be due to alterations in satellite cell differentiation as captopril treatment maintained the mRNA expression of myoblast proliferative markers (Myf5, Pax7) and decreased the expression of myogenin (a marker of differentiation). This was in agreement with IHC staining demonstrating that captopril treatment induced a strong trend ($p=0.06$) for a decrease in the proportion of myogenin+ (differentiation) myoblasts. Furthermore, this also resulted in a delay in muscle fibre maturation as captopril treatment maintained the expression of the embryonic isoform of myosin heavy chain (eMHC). Collectively these findings implicate the activation of a local

angiotensin signaling system as a necessary component of skeletal muscle fibre formation, myonuclear accretion and satellite cell function.

**Introduction**

The growth and maintenance of skeletal muscle mass is largely dependent on a population of myogenic progenitor cells referred to as satellite cells (19). Under basal conditions these cells are maintained in a state of quiescence in the satellite cell niche between the basal lamina and sarcolemma (19). However, in response to myotrauma these cells become activated, enter the cell cycle and proliferate, giving rise to an expansion of the myogenic precursor pool (10). These myoblasts then migrate to the site of injury and subsequently differentiate to form new nascent myotubes or fuse with existing fibres to support repair and growth of skeletal muscle (10). It is now well documented that the genesis of new fibres proceeds in a step wise fashion with the initial fusion occurring between myoblasts to form a nascent myotube containing few nuclei and is followed by the fusion of myoblasts to existing myotubes to induce its growth (11, 12).

The process of a quiescent satellite cell becoming activated, dividing, migrating and differentiating is termed the myogenic program and is controlled by a set of transcriptional networks known as the myogenic regulator factors (Myf5, MyoD, MRF4 and myogenin) as well as the paired box transcription factor Pax7(3). Although these transcriptional regulators have been well characterized,

very little is known regarding the upstream signals that serve to activate and repress their function. We have recently identified a local angiotensin II (Ang II) signaling system in cultured primary and C2C12 myoblasts (14). The presence of this system was defined by the expression of angiotensinogen, angiotensin converting enzyme (ACE), angiotensin type 1 and 2 (AT1, AT2) receptors and alternative processing enzymes, including cathepsin D (14). We also demonstrated that cultured C2C12 cells possess the ability to locally secrete both angiotensin I and ang II *in vitro* as identified by liquid chromatography mass spectrometry (LC-MS) (14).

The function of Ang II in skeletal muscle is not well understood. We have recently demonstrated that inhibition of Ang II signaling (through captopril treatment or AT1 receptor ablation) impairs the growth of skeletal muscle following cardiotoxin (CTX)-induced injury (PLoS One, in review). Furthermore, we have demonstrated that Ang II can specifically act on satellite cells, inducing their activation and chemotaxis (PLoS One, in review). These findings are in agreement with Gordon and colleagues (7) and Westerkamp and colleagues (29) who demonstrated, using models of overload induced compensatory hypertrophy, that ACE inhibition significantly represses skeletal muscle hypertrophy evidenced by a decrease in total protein content fibre cross-sectional area (CSA) and an inability to accrete new fibre nuclei.

Although we have described a role for Ang II in regulating the early response of satellite cells to muscle injury, Ang II may also play an important role

in other satellite cell processes related to activation and chemotaxis. Indeed, Ang II signaling has been shown to regulate numerous cellular effects in many tissues such as proliferation, protein synthesis (13, 17), protein degradation (31) and survival (22). In this study we further define skeletal muscle as possessing a local Ang II signaling system that functions to regulate myonuclear addition and hyperplasia following severe myotrauma. These results highlight the capacity for Ang II to act pleiotropically in skeletal muscle and muscle stem cells.

**Materials and Methods**

*Animals/experimental procedures.* Two experiments (exp 1-2) were performed. Ten-week-old C57Bl/6 mice (n=10 per group (exp1), n=5 per group (exp2), Jackson laboratories, USA) were supplemented with either normal drinking water or captopril (0.5mg/mL, Sigma, Canada) treated drinking water three days prior to and throughout the experimental protocol. Animals were subjected to bilateral injections of CTX (25µl at 10µM) into the TA muscle and tissues were harvested 10 and 21 days (exp1) or 7, 10 and 21 days (exp2) post injection. Also, for reference of normal skeletal muscle architecture, a non-injured, non-supplemented group (n=8) was included. All procedures were conducted according to guidelines established by the Canadian Council on Animal Care with ethics approval from the McMaster University Research Ethics Board.

*Histology.* Skeletal muscles were excised and fixed in neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) to reveal

skeletal muscle architecture. Stains were viewed on a Nikon Eclipse 90i and the number of muscle fibre associated nuclei was calculated by analyzing 300 fibres per animal using Nikon NIS Elements 3.0 software. Also, the total number and density of fibers (fibers/$\mu m^2$) was analyzed 10 and 21d post injury in the TA muscle.

*RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction*. RNA was isolated from control and captopril treated mice 10, and 21d post CTX injection using the RNeasy method according to the manufactures instructions (Qiagen Sciences, Montreal, Canada) and analyzed using quantitative RT-PCR (qRT-PCR). Gene expression fold change was calculated using the delta-delta Ct method (18) using ribosomal protein L32 as a housekeeping gene as its expression was not different between control and captopril treated groups. Primer sequences can be found in Table 1.

*Immunohistochemistry*. TA cross-sections were cleared of paraffin by treatment with xylenes and rehydrated with graded ethanol washes. Epitopes were then retrieved by boiling sections in 10mM tri-sodium citrate buffer for 25 min at 94°C. Sections were then blocked in 5% goat serum for 1.5h or and incubated with primary antibodies to AT1 and pan-angiotensin that recognizes angiotensinogen, Ang I and Ang II (SC-1173, -9040, -7419, Santa Cruz Biotechnologies, USA) overnight at 4 °C. Sections were then incubated in biotinylated anti-goat secondary antibody (1:200, Vector Laboratories, Canada) followed by visualization using the ABC-immunoperoxidase method (PK-6100, SK4100

Vector Laboratories, Canada) and counterstained with Mayers hematoxylin. For Pax7 and myogenin staining (neat hybridoma supernatants, Developmental Studies Hybridoma Bank, USA), sections were stained using the Vector mouse-on-mouse kit as per the manufactures instructions with minor modifications (overnight blocking) and visualized using the ABC-immunoperoxidase method. The number of Pax7+ or myogenin+ cells expressed per 100 fibres were analyzed by counting 250+ fibres respectively using a Nikon Eclipse 90i.

**Results**

*CTX-induced injury activates a local skeletal muscle angiotensin signaling system.* We have previously demonstrated that cultured skeletal muscle myoblasts and myotubes possess a local angiotensin signaling system with the ability to produce Ang II in culture (14). Therefore, we assessed the response of components of this local system to muscle damage *in vivo* using IHC. Under basal conditions AT1 and angiotensin (pan-angiotensin antibody) display weak, diffuse staining suggesting low protein expression (Fig 1A, C). However, 7 days following injury robust staining of AT1 and angiotensin was observed in newly regenerated fibers (displaying central nuclei) showing a clear activation of this local system (Fig 1B, D). Interestingly, as regeneration proceeds, AT1 is downregulated resulting in a mosaic staining pattern whereby some fibres remain strongly positive while others are devoid of positivity (Fig 1E, F). These results

suggest that Ang II signaling through the AT1 receptor may play a role in the regenerative response to muscle injury.

*Captopril treatment inhibits myonuclear accretion and induces hyperplasia.* Based on the IHC results and our previous findings that inhibition of Ang II signaling resulted in decreased myofibre size following injury (PLoS One, in review), we then investigated the role of Ang II signaling in the formation and accretion of nuclei into myofibres during regeneration. Analysis of H&E stained cross-sections demonstrated that when compared to controls ACE inhibition significantly reduced the myonuclei/fibre ratio by 28%, 21d following CTX injury (Fig. 2C). No differences were observed 10d following injury (data not shown). Interestingly, when the total number of myofibres per TA was analyzed, it was discovered that captopril treatment also induced substantial hyperplasia with a 37% increase in total fibre number compared to controls ($p<0.05$, Fig. 2D) 21d following injury while no differences were observed 10d following injury (data not shown). This also translated into a 56% increased density of myofibres/$\mu m^2$ (Fig. 2E). Therefore, while controls appeared to reach a plateau in fibre number by 10d, the captopril treated mice continued to form myofibres until day 21. Collectively, these results indicate that a lack of Ang II signaling during muscle regeneration results in the synthesis of more myofibres that contain fewer myonuclei.

*Blockade of Ang II production alters MRF gene expression.* The formation and growth of myofibres following injury is dependent on many aspects of satellite cell

function including adequate expansion of the satellite cell pool, efficient differentiation of myoblasts and the subsequent maturation of *de novo* fibres (10). Therefore, we assessed the mRNA expression of the MRFs and the content of satellite cells 10 and 21d following CTX injury in control and captopril treated mice. 10 days following injury, captopril treatment appeared to retain the expression of satellite cell proliferative markers with a significant 1.7 and 1.9 fold increase in Myf5, and Pax7 (Fig. 3A, B) respectively, while repressing the expression of markers of differentiation (myogenin – 2.9 fold decrease; $p<0.05$, Fig. 4E). We believe this gene expression profile is indicative of an inhibition of satellite cell differentiation/fusion as the majority of myoblasts should be undergoing terminal differentiation and fusing to myofibres to increase their myofibre CSA and nuclear content.

*ACE inhibition decreases satellite cell content following CTX-induced injury.* Based on the qRT-PCR data we conducted IHC staining of satellite cell populations to assess the effect of captopril treatment on muscle stem cell content. Although no significant differences were observed in total satellite cell content (Pax7+ cells, data not shown), a strong trend ($p=0.06$) for a reduction in the proportion of myogenin+ (differentiating) myoblasts was evident with captopril treatment 10d post CTX injection (Fig. 4D) in agreement with the myogenin expression analysis. To lend further support to the idea that captopril treatment induced delayed differentiation we assessed the expression of the embryonic isoform of myosin heavy chain (eMHC) as this isoform is only expressed in newly

formed fibres and is rapidly downregulated (4). qRT-PCR analysis revealed a significant 3.5-fold increase in eMHC with captopril treatment in comparison to controls (Fig. 3D) 10d post CTX injection demonstrating that the inhibition of myoblast differentiation resulted in delayed fibre maturation. We then examined whether captopril affected the basal content of satellite cells following the repair process of 21 days. The proportion of Pax7+ cells following captopril treatment decreased by 25% as compared to controls (Fig. 5D). In agreement, we also observed a significant reduction in the expression of MyoD (Fig. 3C) at the same timepoint. Collectively these results suggest that the observed reduction in myonuclei/fiber and altered fiber formation observed 21d following injury with captopril treatment is due to alterations in satellite cell content and function.

**Discussion**

In the present manuscript we report that skeletal muscle possess a local Ang II signaling system that functions to regulate muscle fibre formation, myonuclear addition and fibre maturation during *in vivo* mammalian muscle regeneration. The myogenic effects of Ang II appear to be related to its regulatory role of satellite cell content and differentiation. In addition to our previous reports, these findings implicate Ang II as pleiotropic in skeletal muscle independent of its characterized role in regulating blood pressure and fluid homeostasis.

We have extended our previous findings demonstrating that isolated primary and C2C12 myoblasts and myotubes express a local Ang II signaling system that is responsive to mechanical stimulation (14). Local injection of CTX, and the subsequent regenerative response, clearly induced the activation of key members of this local system, the exact function of which is not fully understood. Nevertheless, we report an increased production of angiotensinogen, and/or its metabolites, at the onset of regeneration using immunohistochemistry. The absence of angiotensinogen in uninjured muscle followed by a robust upregulation and progressive downregulation over the regeneration time course suggests that the intramuscular RAS is not ubiquitously active under basal conditions and requires an appropriate cue for activation. This notion was further supported by the observation that the AT1 receptor was expressed in the same temporal fashion following injury. An intriguing observation was the mosaic staining pattern of AT1 observed 21 days following injury and was reminiscent of a fibre-type distribution stain. Regrettably, methodological complications (formalin fixation) precluded the ability to perform fibre-type analysis; however AT1 fibre-type specificity remains an important question that requires further investigation. Interestingly, the activation pattern of both angiotensin and AT1 were similar with the most intense staining patterns observed early following the genesis of new fibres. At this time, the regulatory relationship between skeletal muscle angiotensin and AT1 expression is currently unknown but it is possible that feed-forward mechanisms may drive the increased production of Ang II to

further aid in the regenerative response. Indeed, feed-forward mechanisms have been described in hepatocytes, whereby Ang II induces nuclear factor-K B signaling and subsequent angiotensinogen promoter activity and transcription (16).

Previous *in vitro* observations by our lab identified Ang II as a potent chemotactic agent (PLoS One, in review). Based on these observations we hypothesized that the increased production of angiotensin during regeneration serves, at least in part, to act as a migratory stimulus to attract myoblasts to fuse with a growing fibre. Since the growth of skeletal muscle is partially contingent upon the addition of new nuclei to the myofibre (9), we assessed the effect of ACE inhibition on nuclear addition as well as myofibre formation and satellite cell function. Here we demonstrate that Ang II is necessary for normal myonuclear accretion in myofibers during muscle regeneration. These results are in agreement with Westerkamp and colleagues who demonstrated, using a synergist model of overload induced hypertrophy (29), that ACE inhibition resulted in the inability to add new nuclei to the soleus muscle. Collectively, these findings highlight the function of ACE as a focal point of regulation on skeletal muscle size and nuclear content. Along these lines, recent investigations have identified a polymorphism of a 287-base-pair insertion (I) in the ACE gene and the absence of this insertion (deletion – (D)) is associated with higher plasma ACE activity (23). Interestingly, associations between the ACE genotype and physical performance have been observed with the homozygous expression of

the D allele being correlated with enhanced training-related strength gains while homozygous expression of the I allele has been linked with improved endurance performance (15, 30). Although these reports remain highly controversial, they identify ACE as the only known gene polymorphism linked to physical performance.

Perhaps most surprisingly, we observed a significant increase in the total number of fibres in the TA following 21 days of regeneration with captopril treatment. Skeletal muscle fiber hyperplasia has been previously induced using load-induced stretch of avian skeletal muscle (1, 2) as well as "strength training" of cats (5, 6) and rodents (27) while no reports exist documenting the induction of hyperplasia due to severe injury. To our knowledge this is the first report describing the induction of hyperplasia due to captopril treatment during CTX-induced injury. The functional significance and molecular explanation of this captopril-induced hyperplasia is currently unknown. However, it is conceivable that the previous observations of repressed myoblast chemotaxis due to inhibited Ang II signaling (PLoS One, in review) and impaired cell differentiation observed in the present study (see below) could collectively result in hyperpalsia. For example, if satellite cells are not able to efficiently migrate and fuse with *de novo* formed fibres, there is potential that aberrant cell to cell fusion could occur resulting in the production of more fibres. This hypothesis is in agreement with the *in vivo* IHC staining demonstrating the increased production of angiotensin following injury. We propose that this may serve as the chemotactic signal

directing the differentiation of myoblasts and subsequent growth of myofibres. Although potentially considered a beneficial adaptation, it should be noted that under the current experimental conditions hyperplasia was accompanied by an inability to add nuclei to existing fibres, inhibited maturation of myofibres and disrupted satellite cell response. Therefore, the functional relevance of this observation requires further investigation.

Although the exact molecular mechanisms underlying altered fibre formation and nuclear accretion are not understood, it is likely that central to this phenotype is the repression of satellite cell function. First, an adequate expansion of satellite cells is necessary to provide a sufficient source of nuclei to fuse with the growing myofibers (3). This may potentially be limited with inhibited Ang II signaling as captopril treatment significantly decreased the proportion of Pax7+ satellite cells *in vivo* 21d following CTX-induced injury. However, following 21 days the regenerative response is largely complete and this finding may be more indicative of the function of captopril in regulating the maintenance of the satellite cell pool. Furthermore, no differences were observed in the number of Pax7+ cells 10 days following injury, a more relevant time-point during muscle regeneration. Therefore, a more likely explanation is that captopril treatment inhibits the ability of satellite cells to fuse with existing myofibres. This theory is supported by both qRT-PCR and IHC staining data indicating that captopril inhibits markers of satellite cell differentiation, decreases the number of differentiating myoblasts while maintaining the expression of proliferative

markers. How Ang II may function to regulate myoblast differentiation is currently unknown. However, Ang II has been demonstrated to activate signaling pathways necessary for myoblast differentiation in other cell types. For example, Ang II signaling in cardiomyocytes has recently been demonstrated to increase the activation and binding of members of the nuclear factor of activated T-cells (NFAT) family (20, 25, 26) which have been implicated as indispensable in regulating myoblast fusion (21). Moreover, Ang II increases the mRNA and protein content of vascular cell andhesion molecule-1 (VCAM-1) in rat aortic smooth muscle cells (28) which is expressed on sites of secondary myogenesis *in vivo* and regulates myoblast differentiation in culture (24).

Clinically, ACE inhibitors and angiotensin receptor blockers (ARBs) are amongst the most commonly prescribed antihypertensive medications (8). Although generally considered safe, few studies have examined the direct effects of ACE inhibitors or ARBs on skeletal muscle function, adaptation to exercise or muscle regeneration in humans. Therefore, further investigation is warranted to delineate all potential secondary effects of these widely prescribed pharmaceuticals.

In summary, the current investigation provides evidence that the activation of a local Ang II signaling system is a necessary component of skeletal muscle fibre formation and nuclear accretion following CTX-induced injury. Furthermore, this local system functions to regulate the differentiation of muscle satellite cells and maturation of *de novo* myofibres.

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**Tables:**

**Table 1. Primer sequences used**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| Myf5 | TGAAGGATGGACATGACGGACG | TTGTGTGCTCCGAAGGCTGCTA |
| MyoD | TACCCAAGGTGGAGATCCTG | CATCATGCCATCAGAGCAGT |
| Myogenin | CTACAGGCCTTGCTCAGCTC | AGATTGTGGGCGTCTGTAGG |
| Pax7 | CTGGATGAGGGCTCAGATGT | GGTTAGCTCCTGCCTGCTTA |
| eMHC | AAAAGGCCATCACTGACGC | CAGCTCTCTGATCCGTGTCTC |
| L32 | TCCACAATGTCAAGGAGCTG | ACTCATTTTCTTCGCTGCGT |

**Figure legends**

**Figure 1. CTX injection activates a local angiotensin signaling system.** Representative images of IHC staining of angiotensin (pan) in uninjured (A) and regenerating (7d post CTX, B) skeletal muscle (4x magnification) with secondary only controls (7d post CTX, G, 20x magnification). Representative images of IHC staining of AT1 in uninjured (C) and regenerating skeletal muscle 7 (D), 14 (E) and 21 (F) days following CTX injection (40x magnification) with secondary only controls (7d post CTX, H, 20x magnification).

**Figure 2. Captopril treatment inhibits myonuclear accretion and induced hyperplasia.** Representative H&E stains of the TA of control (A) and captopril treated (B) mice 21 days following CTX injection. C) Analysis of the nuclei/fibre ratio (D), the total number (E) and the density of fibres within the TA of control and captopril treated mice 21 days following CTX injection.

**Figure 3. ACE inhibition alters MRF gene expression following injury.** qRT-PCR snalysis of the expression of Myf5 (A), Pax7 (B), MyoD (C) and eMHC (D) 10 and 21 days following CTX injection.

**Figure 4. Captopril treatment inhibits myogenin expression**. Representative IHC stains of myogenin 10 days following CTX injection within the TA of control (A) and captopril (B) treated mice with (C) representing secondary negative

control staining. D) Analysis the proportion of myogenin+ cells 10 days following injury. E) qRT-PCR analysis of the expression of myogenin in control and captopril treated mice 10 and 21 days following CTX injection.

**Figure 5. ACE inhibition decreases *in vivo* satellite cell content**. Representative IHC stains and analysis (D) of the proportion of Pax7+ within the TA of control (A) and captopril treated (B) mice 21 days following CTX injection with (D) representing secondary negative control staining.

**Figures.**

**Figure 1.**

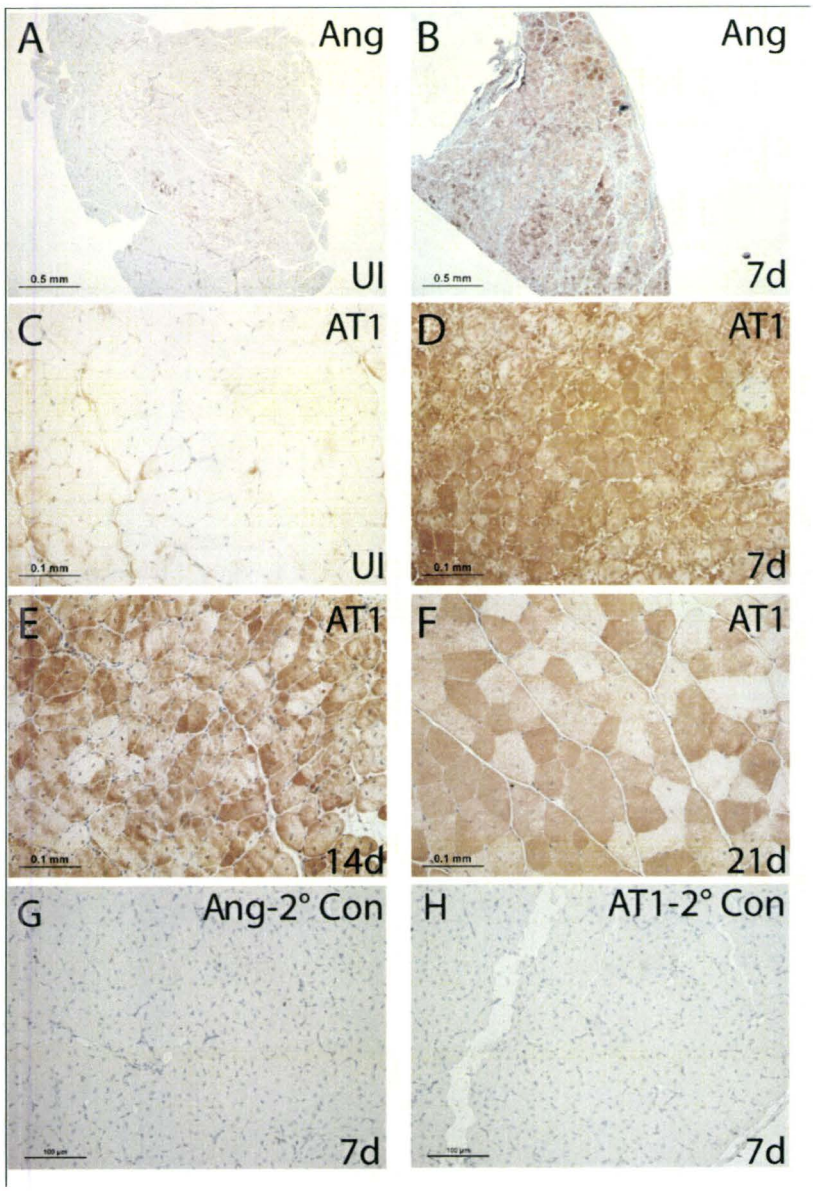


**Figure 2.**

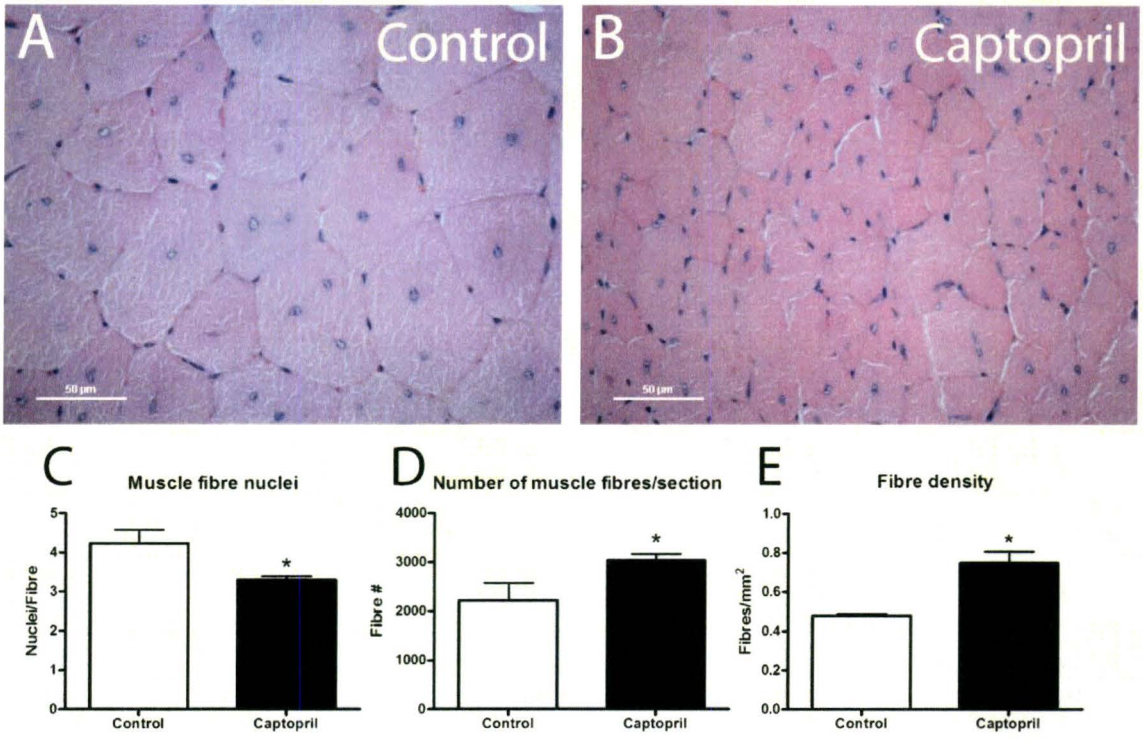

A
Control
50 µm
B
Captopril
50 µm
C
Muscle fibre nuclei
Nuclei/Fibre
0
1
2
3
4
5
*
Control
Captopril
D
Number of muscle fibres/section
Fibre #
0
1000
2000
3000
4000
*
Control
Captopril
E
Fibre density
Fibres/mm2
0.0
0.2
0.4
0.6
0.8
1.0
*
Control
Captopril

**Figure 3.**

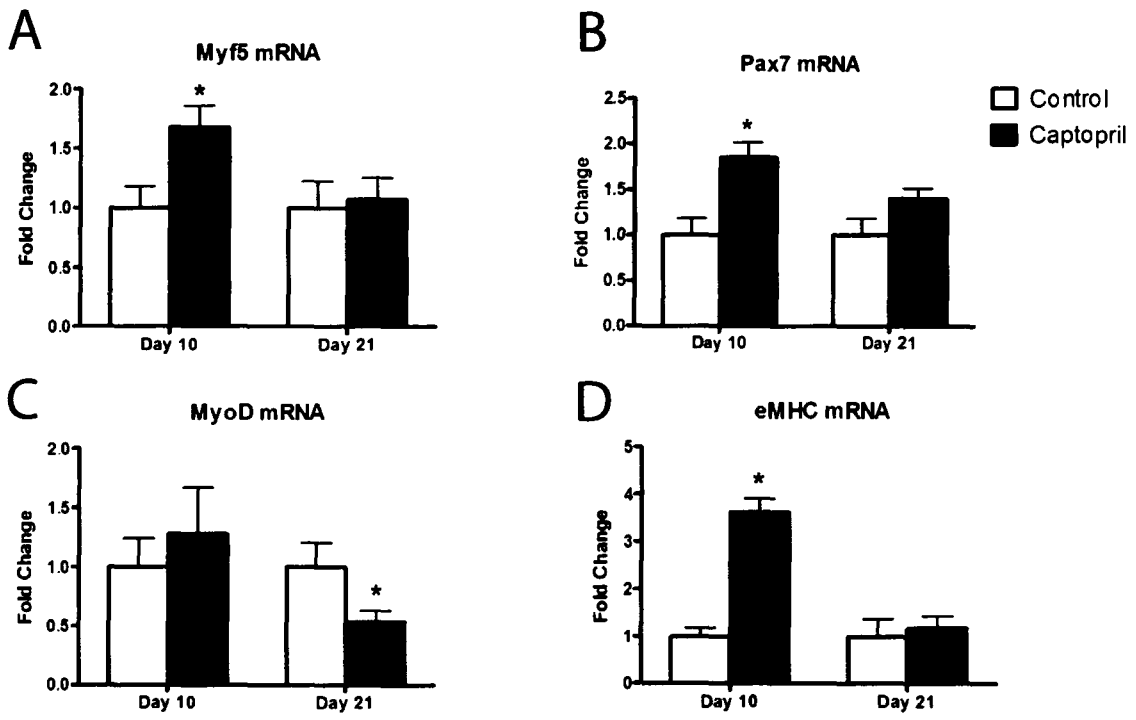

A
Myf5 mRNA
Fold Change
2.0
1.5
1.0
0.5
0.0
*
Day 10
Day 21
B
Pax7 mRNA
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*
Day 10
Day 21
Control
Captopril
C
MyoD mRNA
Fold Change
2.0
1.5
1.0
0.5
0.0
*
Day 10
Day 21
D
eMHC mRNA
Fold Change
5
4
3
2
1
0
*
Day 10
Day 21

**Figure 4.**

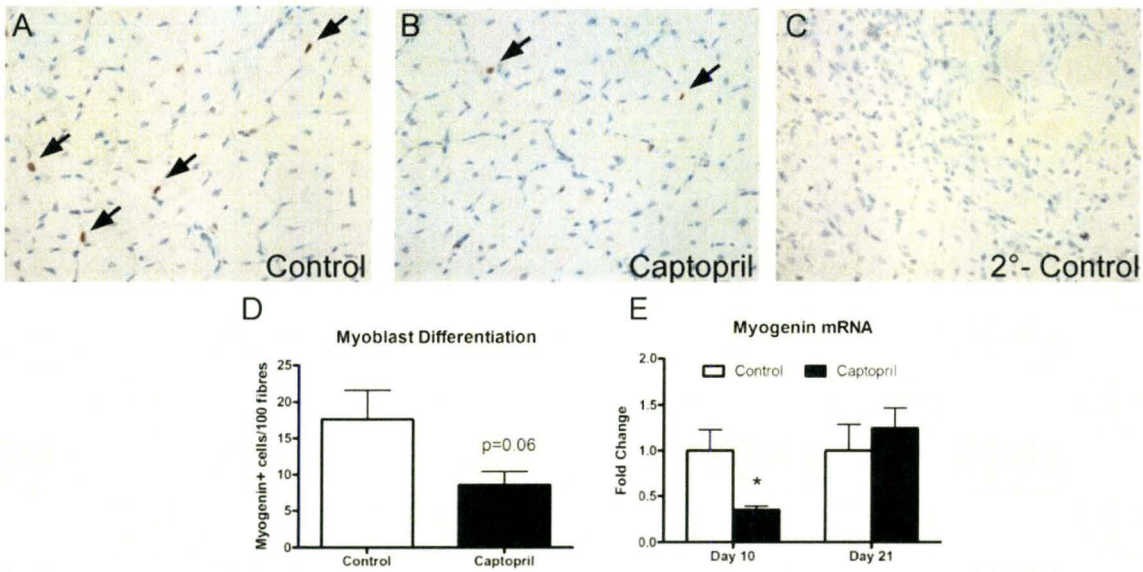
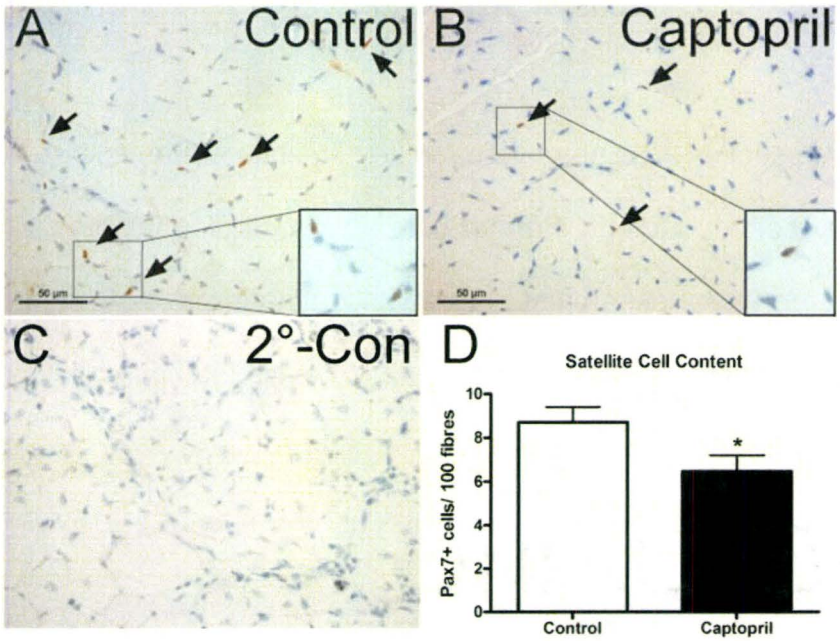

A
Control
B
Captopril
C
2°- Control
D
Myoblast Differentiation
Myogenin+ cells/100 fibres
25
20
15
10
5
0
p=0.06
Control
Captopril
E
Myogenin mRNA
Control
Captopril
Fold Change
2.0
1.5
1.0
0.5
0.0
*
Day 10
Day 21

**Figure 5.**


A
Control
B
Captopril
50 µm
50 µm
C
2°-Con
D
Satellite Cell Content
Pax7+ cells/ 100 fibres
10
8
6
4
2
0
*
Control
Captopril

# Chapter 5

# General Discussion

## 5.1 Introduction

The following section will summarize the key findings of the studies described in the present document. Furthermore, this section will outline recommendations for future research studies investigating the role of Ang II in regulating the regeneration, growth and maintenance of skeletal muscle.

## 5.2 Mechanical stretch alters the expression of members of the RAS within skeletal muscle myoblasts and myotubes.

Early studies have provided evidence for the presence of a local RAS in skeletal muscle *in vivo*. These investigations demonstrated that skeletal muscle possesses ACE activity [1], the AT1 and AT2 receptors [2, 3] and the capacity to produce Ang II [4]. However, until now it was thought that the local RAS in skeletal muscle was found only in the microcirculation of the vascular bed and not within the skeletal muscle fibres themselves. Furthermore, no studies have investigated the potential for skeletal muscle stem cells to possess components of the RAS even though such as system has been demonstrated in other stem cell compartments such as bone marrow [5]. Here, we are the first to describe that cultured muscle stem cells and myotubes differentially express members of the RAS and possess the ability to secrete Ang II. Interestingly, the genesis of Ang II in skeletal muscle is not dependent on the activity of renin since renin was

not expressed in C2C12 cells, primary myoblasts or whole skeletal muscle. Although we have identified candidate enzymes with renin-like capacity, future studies should identify and further describe which enzymes act on angiotensinogen produced by muscle cells both *in vitro* and *in vivo*. Interestingly, we also demonstrate differential localization of the AT1 and AT2 receptors in myoblasts and myotubes. Although AT2 receptors appeared to be expressed ubiquitously on the cell, AT1 was found almost exclusively in the nuclei of differentiated myotubes and colocalized with polymerized active filaments in myoblasts. The functional significance of these findings remains to be elucidated but may speak to the specific function of AT1 in regulating differential processes such as the activation and migration of myoblasts. .

Mechanical stimulation is a potent activator of the cardiac RAS [6] and a key regulator of satellite activation and proliferation [7]. Similarly, we describe that cyclic mechanical strain can alter the expression of key members of the RAS in myoblasts and myotubes. Given the current results demonstrating a role for Ang II in activating quiescent muscle stem cells it is attractive to hypothesize that the local angiotensin signaling system within these cells responds to mechanical stretch to activate satellite cells following muscle contraction in an autocrine fashion. Even though we demonstrate a response of these RAS members to stretch, we have not investigated the effect of mechanical stimulation on Ang II production or the mechanisms that sense and propagate this stimulus in muscle cells. Therefore, inquiry into these questions would increase our understanding

of the mechanisms governing this system. Although not fully understood, it has also been recently demonstrated that mechanical stretch of cardiac myocytes can stimulate AT1 mediated signaling independent of Ang II production [8]. Therefore, future investigations should also identify if such a system exists in muscle stem cells and fibres as this would represent a novel mechanism of activation solely based on mechanical stretch, a major component of muscle contraction.

Importantly, we also provide evidence that this local angiotensin signaling system is present *in vivo* and becomes active in response to muscle injury. Using IHC we demonstrate that skeletal muscle fibres increase the production of angiotensinogen and AT1 during myogenesis following myotrauma. These interesting findings should serve as the basis to answer the many basic regulatory questions that remain unanswered regarding the presence and responsiveness of this system. For example, what is the concentration of Ang II in skeletal muscle basally and in response to muscle injury? Similarly, does the skeletal muscle angiotensin signaling system act independent or in concert with the systemic RAS? Do the local angiotensin signaling systems in satellite cells and mature myofibres interact? What enzymes act on angiotensinogen and function in place of renin *in vivo*? Moreover, what other stimuli (i.e. exercise, pathology) activate the skeletal muscle angiotensin signaling system. It is clear that numerous in depth inquires are required to delineate the answers to these many questions.

## 5.3 Future research into RAS regulation: Ang 1-7, Ang II metabolites and AT1 receptor regulation

Over the past 100 years our understanding of the RAS has expanded significantly. This system is no longer solely considered a systemic cascade controlling fluid dynamics, but rather an integrated system encompassing complex interactions with many organs and local RASs. Interestingly, recent investigations have identified entirely new levels of RAS regulation involving the differential processing of Ang I and II by alternative enzymes and the subsequent stimulation of recently cloned receptors by the resulting metabolites. Specifically, Schiavone and colleagues [9] have identified a bioactive peptide comprised of the first seven amino acids of Ang II, which was subsequently termed Ang 1-7. Ang 1-7 can be formed by the enzymatic degradation of Ang I or from the cleavage of the terminal phenylalenine residue of Ang II. The latter is performed by the recently identified ACE alternative termed ACE2 [10]. Although Ang 1-7 shares the exact same amino acid sequence with Ang II, it does not bind to the AT1 and AT2 receptors. Conversely, it has the capacity to bind to its own G-coupled receptor, Mas [10]. The actions of Ang 1-7 binding to the Mas receptor have not been fully defined but are generally antagonistic to that of AT1-mediated Ang II signaling such that Ang 1-7 is vasodilatory, antiarrhymogenic and anti-proliferative in cardiomyocytes and VSMC [11]. Similarly, the recently identified angiotensin 1-9 (Ang 1-9) has been demonstrated to decrease circulating Ang II

levels, inhibit ACE activity and prevent cardiomyocyte hypertrophy, signaling through an unknown receptor [12]. Interestingly, recent reports have also identified proteins that have the ability to act directly on the AT1 receptor to regulate its function. For example, the AT1 receptor associated protein (ATRAP) has been demonstrated to inhibit normal AT1 receptor recycling by promoting its internalization, which subsequently inhibits actions such as cardiac hypertrophy [13]. Unfortunately, we have not investigated the possibility that members of this extended RAS are also present in skeletal muscle and satellite cells, thus further investigation is warranted.

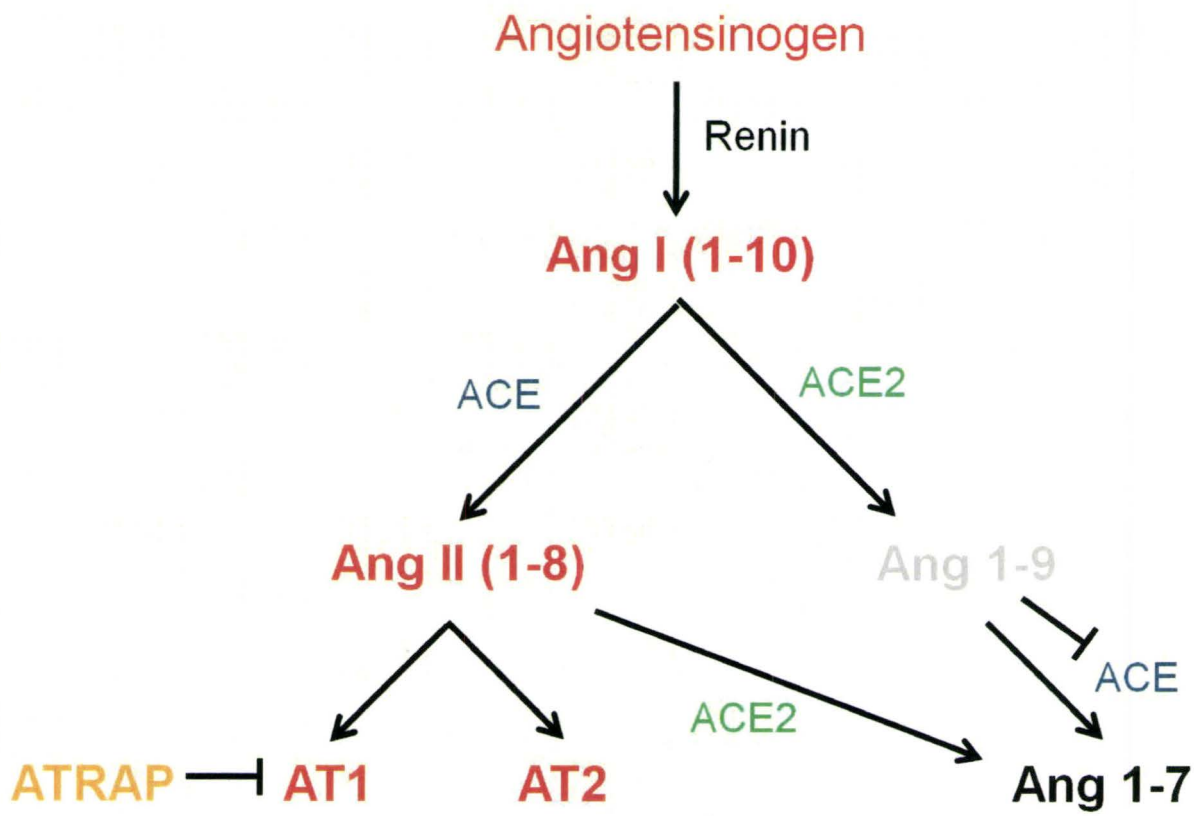



**Figure 1. The extended RAS**. In addition to creating Ang II, Ang I can be cleaved by ACE2 to create Ang 1-9 which can be subsequently cleaved by ACE

to form Ang 1-7. However, the enzymatic degradation of Ang II by ACE2 is the primarily source Ang 1-7 formation which functions to repress the actions of Ang II. This is also accomplished through the binding of ATRAP to the AT1 receptor which promotes it internalization.

## 5.4 Ang II: Regulator of Muscle Fibre Formation and Growth Following Injury

Since we have described the presence of a local angiotensin signaling system in muscle stem cells and cultured myotubes, we wanted to assess if this local system was present *in vivo,* and if so, did it become active during muscle regeneration? We report that severe myotrauma induced the presence of angiotensinogen and the AT1 receptor during myogenesis. Interestingly, both angiotensinogen and AT1 were downregulated as regeneration and growth progressed until a mosaic staining pattern was evident with AT1 expression 21 days following injury. These findings implicate the activation of a local skeletal muscle angiotensin signaling system as a modulator of skeletal muscle growth and regeneration.

To investigate the effect of angiotensin II signaling blockade on muscle growth and regeneration, *in vivo*, we induced muscle injury using CTX injection in TA muscle in mice treated with captopril to inhibit the endogenous production of Ang II or in mice lacking the AT1a receptor. We demonstrate that captopril treatment or the loss of the AT1a receptor significantly inhibited the growth of muscle fibres from days 10-21 following injury. This translated to significant increases in the proportion of small fibres while the proportion of large and

medium size fibres was reduced with ACE inhibition. These findings are in agreement with work from Gordon and colleagues [14] who were the first to identify Ang II as a regulator of skeletal muscle fibre size. Using a model of synergist ablation, they demonstrated that ACE inhibitor treatment blocked the hypertrophy associated with compensatory overload. Importantly they were able to rescue this defect with the administration of local Ang II delivered directly to the muscle via an osmotic pump. We also demonstrate that following CTX injection, captopril inhibited the addition of myonuclei to myofibres. This effect of captopril on myonuclear addition is in agreement with work from Westerkamp and colleagues [15] who demonstrated that ACE inhibitor treatment induced a similar effect using a model of overload induced compensatory hypertrophy. This also coincided with reductions in muscle fibre CSA when compared to controls. Surprisingly, we also demonstrated that captopril treatment induced a significant hyperplasia 21 days following CTX injection.

In an attempt to explain these observations we assessed the involvement of Ang II in regulating muscle satellite cell function as these precursor cells are primarily responsible for coordinating the formation and maturation of skeletal muscle following injury. Using *in vitro* models we demonstrated that Ang II had the capacity to regulate the early response of satellite cells to muscle injury by inducing the activation and chemotaxis of myoblasts. Satellite cell chemotaxis appeared to be regulated by Ang II induced reorganization of the actin cytoskeleton and upregulation of MMP2. Since the processes of satellite cell

activation and chemotaxis are also intimately tied to other satellite cell functions such as differentiation and fusion we also assessed the proportion of differentiating myoblasts and the response of the MRFs in injured skeletal muscle of captopril treated mice. IHC and qRT-PCR analysis revealed that captopril treatment appeared to inhibit the differentiation of myoblasts evidenced by the sustained expression of proliferative markers and a decreased expression of myogenin and a trend for a decrease in the number of myogenin+ cells.

Collectively, we propose the following theoretical model of Ang II regulation of skeletal muscle growth and regeneration based on the findings described in the present document (figure 2). Following myotrauma (and potentially muscle contraction), Ang II is released by the muscle fibre and functions to upregulate MRF gene expression and activate neighboring muscle satellite cells, preparing them for proliferation. The regenerating muscle fibre also increases the expression of the AT1 receptor which interacts with Ang II. Although the precise function for an upregulation in AT1 expression is currently unknown, it is possible that this occurs to drive the expression of angiotensinogen and subsequently Ang II formation. Importantly, Ang II then functions to direct the regenerative response by inducing the chemotaxis of myoblasts to the site of injury by increasing the content of MMP2 and stimulating lamellipodial formation. Ang II then participates in the proper alignment of cells during myoblast differentiation and fusion by regulating migratory kinetics. Therefore, in response to Ang II signaling blockade satellite cell activation and chemotaxis are inhibited

resulting in the inability of myoblasts to undergo normal differentiation/fusion to the growing fibres. This subsequently results in aberrant cell fusion and the hyperplasia of muscle fibres, decreased fibre CSA and nuclear content. However, the confirmation of such a model requires further observation and experimentation.

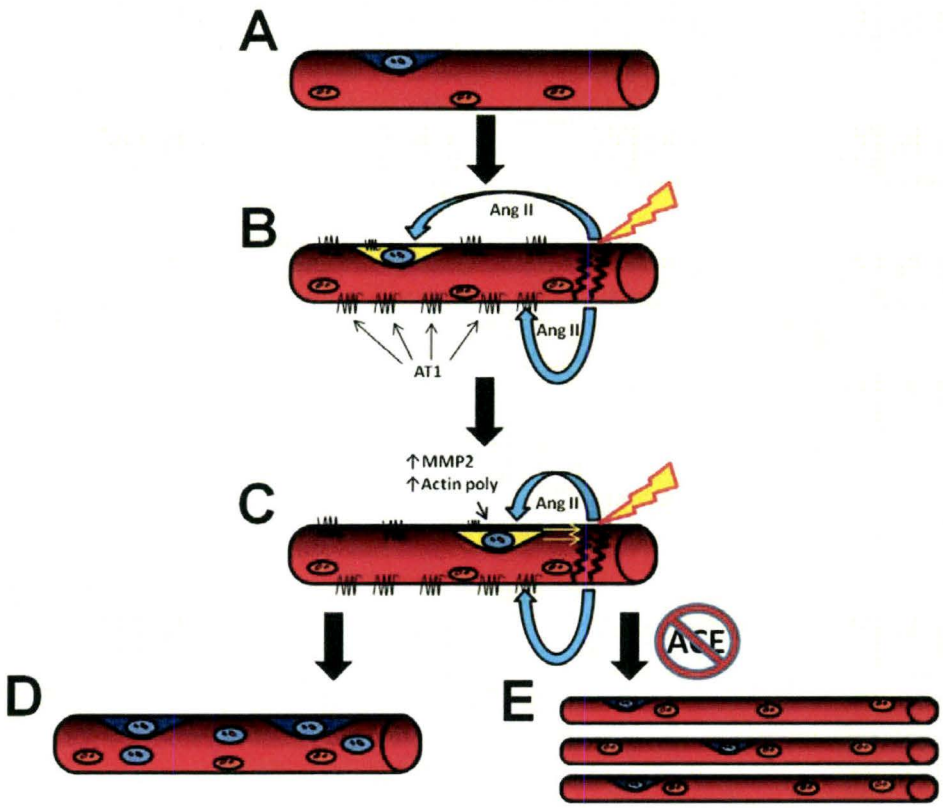



**Figure 2. Theoretical model of Ang II regulation of skeletal muscle growth and satellite cell function during regeneration**. A) Under basal conditions satellite cells sit on the periphery of the myofibre in a state of quiescence. B) In response to myotrauma, Ang II is released by the myofibre and signals the activation of neighboring satellite cells. At this time AT1 becomes upregulated and interacts with Ang II to potentially increase the formation of angiotensinogen in a feed forward loop. C) Ang II then signals the chemotaxis of myoblasts to the site of injury through the increased activity of MMP2 and cytoskeletal reorganization. D) Ang II also functions to direct the proper fusion of myoblasts with the growing fibre through it regulation of cell migration. E) In the absence of

Ang II signaling, abnormal cell fusion occurs which results the hyperplasia of small myofibres with low nuclear content.

## 5.5 Future developmental studies into Ang II function within skeletal muscle and muscle stem cells

The *in vitro* and *in vivo* findings described in this document provide the framework for investigating the molecular mechanisms underlying the Ang II regulation of satellite cell function and skeletal muscle mass. Specifically, what are the signaling cascades that govern the Ang II-induced activation and chemotaxis of satellite cells? Interestingly, it is well known that the binding of Ang II to its receptor results in a significant production of reactive oxygen intermediates (ROIs), which function as signaling molecules increasing the expression of immediate early genes (c-fos, c-jun) and in turn DNA binding of the activator protein-1 (AP-1) complex [16, 17] in C2C12 cells. Similarly, we also demonstrate that Ang II treatment of quiescent C2C12 cells induced the increased expression of c-fos. These findings are intriguing as c-fos induction has been demonstrated to be one of the earliest known events during satellite cell activation *in vivo* [18]. It should also be noted that although many growth factors have been shown to regulate the proliferation of activated satellite cells, very few soluble factors have been identified to induce the activation of quiescent satellite cells. Therefore, further investigation to examine the possibility that AP-1 is a downstream target of Ang II signaling is warranted.

Satellite cell chemotaxis is another poorly understood and extremely important component of muscle regeneration and growth. Only recently have we begun to delineate the molecular mechanisms governing satellite cell migration. HGF is the most well described chemotactic factor in muscle and it has been reported that binding through its receptor, c-met, can induce C2C12 migration through the activation of two cytoskeletal adaptor proteins (N-Wasp and Wave) (which are integral to actin cytoskeletal reorganization and function downstream of phosphatidylinositol 3-kinase (PI3K) [19]. We report that Ang II stimulates myoblast migration through increased MMP2 content and reorganization of the actin cytoskeleton due to an unknown mechanism. The regulation of actin cytoskeletal dynamics is complex and influenced by many factors. In other cell types Ang II has been reported at activate proteins involved in the control of actin cytoskeletal dynamics including RhoA [20, 21] paxillin, tensin and focal adhesion kinase (FAK) [22]. However, further studies are required to pinpoint the exact signaling molecules activated by Ang II in regulating the actin cytoskeleton in satellite cells.

As previously mentioned, satellite cell function is integrated and dynamic, therefore the process of myoblast migration is also intimately tied to many other cellular functions including myoblast fusion. Our *in vitro* and *in vivo* findings suggest that both satellite cell migration and differentiation are impaired by inhibited Ang II signaling. Therefore, we hypothesize that the observed decrease in fibre CSA may be due to impaired myoblast differentiation secondary to

impaired cell migration. More work is required to fully understand this phenomenon such as an examination of myoblast differentiation kinetics in the presence or absence of Ang II.

## 5.6 Future applied studies into Ang II function within skeletal muscle and muscle stem cells

The current document provides evidence that a local angiotensin signaling system exists in skeletal muscle, and associated stem cells, and functions to regulate muscle fibre size and number. However, a question that remains unanswered is whether our observations result in any functional deficits. This should be a central issue for future studies on Ang II in skeletal muscle. Specifically, initial studies should focus on animal models involving both synergist ablation-induced muscle hypertrophy as well as CTX-induced muscle regeneration. Analysis of force production and fatigability using electrical stimulation should be a focal point of these investigations. Analyses following synergist ablation will reveal if the decrease in muscle fibre CSA observed with ACE inhibition translates into a loss of function. Performing a similar analysis in animals undergoing muscle regeneration will provide a functional assessment of the regenerative process and a time-course of the re-acquisition of force generating capacity. Similarly, this analysis will reveal if the hyperplasia of small fibres observed with captopril treatment results in a fatigue resistant phenotype and an oxidative shift towards type-I muscle fibres. If such results are observed,

myosin ATPase fibre-typing should be conducted to confirm the oxidative nature of these fibres. Finally, given the observed mosaic staining pattern of AT1 (which was reminiscent of fibre-type staining), IHC costaining of AT1 with MHC isoforms should be conducted to reveal if AT1 expression is fibre-type specific and therefore implicated as a regulator of fibre type determination.

The use of *in vitro* and animals models has allowed us to delineate some of the cellular and molecular signals involved in the Ang II-induced regulation of skeletal muscle growth. However, the investigation of the *in vivo* role of Ang II in human skeletal muscle growth is a priority. Initial studies should focus on three objectives: 1) Does a local angiotensin signaling system exist in human skeletal muscle and is it responsive to acute exercise? 2) What is the function of Ang II in regulating the acute satellite cell response following a single exercise bout? 3) Is Ang II necessary for the size and strength gains associated with chronic resistance exercise? These objectives should be investigated using two populations: healthy young individuals and the elderly.

To complete these objectives, Ang II signaling should be blocked by supplementing individuals with losartan (AT1 blocker). The analysis of young healthy individuals will allow us to determine under non-pathological conditions if Ang II is a modulator of human skeletal muscle mass and satellite cell function. Acute damaging exercise has been demonstrated to activate satellite cells and initiate the regenerative response [23]. Therefore, these conditions are ideal to assess if a local angiotensin signaling system exists in human skeletal muscle

and if it is responsive to muscle damage. Analysis should include qRT-PCR, IHC and western blotting to identify and quantify members of the RAS in skeletal muscle biopsies pre and post exercise. Furthermore, losartan treatment of young individuals undergoing damaging exercise will delineate if Ang II is a regulator of the early satellite cell response to muscle injury. IHC quantification of satellite cell content and analysis of the MRFs should be undertaken to analyze and delineate the effect of AT1 receptor blockade on the muscle stem cell response. Finally, to assess if Ang II regulates the chronic adaptation of skeletal muscle to resistance exercise, young individuals should be treated with losartan and undergo a chronic, progressive 12-16 week resistance exercise program. Biochemical analysis shoud involve the use of fibre-typing, MRI and functional testing. Results from this investigation will provide definitive evidence of a mechanistic role for Ang II and a local Ang II signaling system in the regulation of human skeletal muscle fibre size, function and composition.

The proposed investigations should also be performed in elderly individuals currently medicated with ACE inhibitors or ARBs. This analysis will shed valuable light on the controversy that currently exists regarding the effect of these pharmaceuticals on skeletal muscle function and adaptation. For example, we and other have demonstrated that ACE activity appears necessary for skeletal muscle growth and hypertrophy using animal models. However a recent report analyzing the decline in strength and walking speed over 3 years in elderly individuals revealed that ACE inhibitor treatment halted or slowed this age-

associated decrease [24]. However, the mechanisms governing these effects are unknown and could very likely be due to secondary vascular events. Furthermore, no studies have assessed the effects of pharmaceutical inhibition of Ang II on skeletal muscle adaptation during resistance exercise in the elderly. Similarly, no studies have examined the effect of Ang II signaling inhibition on the satellite cell response to exercise in an elderly population.

The need for investigation of the secondary effects of ACE inhibitors and ARBs is highlighted by the fact that hypertension is currently one of the most commonly treated pathologies in the elderly [25]. Therefore, the prescription of ACE inhibitors and ARBs for the treatment of this disease has steadily increased and is now considered the first line of defense therapy [26]. Consequently, the research, manufacturing, marketing and sale of these drugs represents a multi-billion dollar industry. Unfortunately, the elderly are also the population most susceptible to the age related decline in muscle mass and function known as sarcopenia. Therefore, delineating the biochemical, molecular and functional impact of Ang II blockade in this population is of significant importance.

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