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Cellular and Molecular Changes Following Skeletal Muscle Damage: A Role for NF- κ B and Muscle Resident Pericytes

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CELLULAR AND MOLECULAR CHANGES FOLLOWING SKELETAL
MUSCLE DAMAGE: A ROLE FOR NF- κ B AND MUSCLE RESIDENT
PERICYTES

A Dissertation Presented

By

ROBERT D. HYLDAHL

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Department of Kinesiology

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Dedicated to my beautiful and committed wife and children

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service to our family. You truly embody everything that is good in this world. I look forward with great eagerness to the next chapter of our lives together.

ABSTRACT

CELLULAR AND MOLECULAR CHANGES FOLLOWING SKELETAL MUSCLE DAMAGE: A ROLE FOR NF- κ B AND MUSCLE RESIDENT PERICYTES

SEPTEMBER 2011

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Skeletal muscle is dynamic and actively regenerates following damage or altered functional demand. Regeneration is essential for the maintenance of muscle mass and, when dysregulated as a result of disease or aging, can lead to losses in functional capacity and increased mortality. Limited data exist on the molecular mechanisms that govern skeletal muscle regeneration in humans. Therefore, the overall objective of this dissertation was to characterize early molecular alterations in human skeletal muscle to strenuous exercise known to induce a muscle regenerative response. Thirty-five subjects completed 100 eccentric (muscle lengthening) contractions (EC) of the knee extensors with one leg and muscle biopsies were taken from both legs 3 h post-EC. The sample from the non-EC leg served as the control. A well-powered transcriptomic screen and network analysis using Ingenuity Pathway software was first conducted on mRNA from the biopsy samples. Network analysis identified the transcription factor NF-kappaB (NF- κ B) as a key molecular element affected by EC. Conformational qRT-PCR confirmed alterations in genes associated with NF-kappaB. A transcription factor ELISA, using nuclear extracts from EC and control muscle samples showed a 1.6 fold increase in NF-

κB DNA binding activity following EC. Immunohistochemical experiments then localized the majority of NF-κB positive nuclei to cells in the interstitium, which stained positive for markers of pericyte cells and not satellite cells. To ascertain the mechanistic significance of NF-κB activation following muscle damage, *in vitro* analyses were carried out using a novel primary pericyte/myoblast co-culture model. Primary pericyte/myoblast co-culture experiments demonstrated that pericytes, transfected with a DNA vector designed to drive NF-κB activation, enhanced proliferation and inhibited myogenic differentiation of co-cultured skeletal muscle myoblasts. Furthermore, reduced NF-κB activation led to enhanced myogenic potential of primary pericytes. Taken together, the data in this dissertation suggest that NF-κB dependent signaling in pericytes regulates myogenic differentiation in a cell- and non-cell autonomous manner and may affect the early regenerative response following muscle damage by inhibiting differentiation and promoting proliferation of muscle satellite cells.

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CHAPTER I

AIMS AND HYPOTHESES

Introduction

Skeletal muscle represents the largest portion of tissue in the human body and is unique among tissues in its remarkable ability to repair and regenerate itself in response to changing functional demands and/or damage. Given its essential roles in locomotion, heat production and energy storage, the regenerative capacity of skeletal muscle is paramount to normal, healthy function. In fact, when a muscle's regenerative capacity is compromised or exhausted, as in the case of aging or muscle degenerative diseases, dysfunction and premature death often result (15). The complex mechanisms that regulate a muscle's ability to regenerate rely on the intricate interactions and signaling patterns between the regenerating myofibers, muscle progenitor cells (MPCs), immune cells and other systemic factors.

Statement of the Problem

There is strong supporting evidence for the activation of muscle regenerative events, as early as 2 hours following a damaging stimulus (4). However, the initial signaling molecules and pathways, as well as the origin of the signals associated with these events following damage are poorly understood, particularly in human muscle. In fact, the majority of studies on muscle regeneration performed to date (and 95% of the studies cited in this review) have been conducted *in vitro* or in animal models. Due primarily to their facilitation of genetic manipulations, these models have provided and will continue

to provide the basis for mechanistic research in muscle regeneration. Nevertheless, given recent emphases for translational research and the possible implications of muscle regenerative work for an aging population, as well as for those afflicted with muscular diseases, future studies will need to specifically address observations made in human muscle. *Therefore, the overall objective of this dissertation will be to further characterize and identify early novel changes in molecular pathways and signaling following muscle damage in humans.*

Experimental Approach

To address the overall dissertation objective, I have taken a translational approach by conducting an unbiased genome wide screen and follow-up analyses in human muscle samples following eccentric exercise-induced muscle damage. These observations were then used to develop mechanistic hypotheses that I tested using an *in vitro* paradigm of myogenesis. Subsequent chapters will present the results of these studies. In Chapter 3 I present a detailed descriptive study, highlighting novel molecular and cellular changes in human muscle following eccentric contractions. Chapter 4 presents the mechanistic resolution of the observations from the human study in the context of muscle regeneration. Additionally, a third published study has been included in the appendix to further underscore the value of using *in vitro* models to elucidate the mechanistic relationships of observations made in human muscle.

Significance

The results of this dissertation will enhance our understanding of muscle regenerative molecular pathways and may provide the basis for development of early interventions following muscle injury. Furthermore, they will also provide valuable mechanistic information on potential molecular targets to drive repair in muscle degenerative diseases such as Duchenne muscular dystrophy.

Specific Aims Study 1: Global Gene Expression Changes Following Eccentric Exercise-Induced Damage

The first step in addressing my dissertation objective of identifying early novel changes in molecular pathways and signaling following muscle damage was to take an exploratory approach by assessing global gene expression changes. I used microarray technology and Ingenuity Pathway software analysis to take a systems approach.

Although others, including our own lab group, have used microarray to assess global gene expression following eccentric exercise (3, 9, 10), small sample size in these studies has always precluded a meaningful systems approach. Also, As noted by the Tumor Analysis Best Practices Working Group (1), the interpretation of microarray generated data can be significantly compromised when the number of replicates are low. Therefore, the primary aims for this dissertation were to:

Specific Aim #1: Use whole genome analysis and a systems analysis to identify genes and pathways that are modified 3 hours following an intense eccentric exercise in a large human subject population.

Hypothesis #1: Whole genome analysis will confirm reports of previously identified differentially expressed genes following eccentric exercise and provide a novel profile of genes and networks that are differentially expressed.

Experimental Design: Muscle biopsies were taken from the vastus lateralis muscle of 35 male subjects 3 h post-100 maximal eccentric contractions (EC). Biopsies were taken from the EC leg and the non-exercised control leg. Muscle damage was then confirmed via measures of strength loss, soreness and serum creatine kinase (CK) pre-EC and for 5 days post-EC. Total RNA was extracted from the control and EC biopsy sample, converted to cDNA, labeled and hybridized to an Agilent Whole Genome Microarray. Raw values from the microarray were log transformed and normalized. Values from the control and EC samples were compared using an ANCOVA and significantly altered genes were input into Ingenuity Pathway Analysis software for systems analysis. qRT-PCR was then used to confirm alterations in genes that were indicated by the microarray analysis.

Brief Results: Indirect markers of muscle damage indicated a moderate level of muscle damage, characterized by the development of soreness, increases in serum CK, and decreases in strength. Ingenuity Pathway Analysis revealed multiple significant gene networks with relevant functions relating to inflammatory and immunologic response, cell growth and proliferation and cell signaling. The transcription factor nuclear factor-kappa-B (NF-kB) was highlighted in several of these networks. Subsequent conformational analysis, using qRT-PCR confirmed alterations in several transcripts associated with NF-kB activation. Collectively, the results of Study 1 suggested that NF-kB activation may be an important molecular response following EC.

Specific Aims Study 2: Activation of NF-kB Following Eccentric Exercise-Induced

Damage

NF-kB is a heterodimeric protein complex that is found in the cytosol in its inactive form. Upon activation, via several well-described mechanisms (2), it is released from its protein inhibitor (Ikb) and can translocate to the nucleus, where it activates transcription of a host of genes. In addition to its most well characterized function as a pro-inflammatory transcription factor, NF-kB is also involved in developmental processes, cell growth, cell proliferation and apoptosis. NF-kB is sensitive to reduction/oxidation reactions and has been shown to be activated by reactive oxygen species (ROS) in several different cell types (7).

Given that EC-induced muscle damage results in a predictable and well-characterized inflammatory and oxidative stress response (11, 14), it would be logical to surmise that NF-kB is activated following EC. In rodents, treadmill running, which has a strong eccentric component, has been shown to increase NF-kB binding activity, enhance Ikk phosphorylation and increase IL-6 mRNA expression, all of which suggest activation of NF-kB (8, 13). Surprisingly, in humans, this observation has never been made. In Study #1, the network analysis indicated possible NF-kB activation following EC in humans. Therefore, the Aims of Study #2 were to:

Specific Aim #1: Determine if NF- κ B is activated in human skeletal muscle following eccentric exercise compared with the non-exercised control leg.

Hypothesis #1: Transcription factor DNA binding analysis and confocal microscopy will show that NF- κ B is activated in skeletal muscle following eccentric exercise compared to non-exercised muscle.

Experimental Design: An ELISA-based transcription factor DNA binding assay was used to assess the activation of NF- κ B in nuclear extracts from the control and EC biopsy samples. Further support for NF- κ B activation was gathered using immunohistochemistry and confocal microscopy. An antibody against the p65 subunit of NF- κ B was used in conjunction with a nuclear stain on serial cross sections of control and EC muscle samples. Confocal images were then acquired and the co-localization of the NF- κ B and nuclear signals indicated the presence of NF- κ B in the nuclei, providing an indirect measure of NF- κ B activity.

Brief Results: By transcription factor ELISA, a significant 1.6 fold increase in NF- κ B DNA binding activity was found in samples from the EC vs. the control leg.

Quantification of the confocal images showed a greater percentage of nuclei that were positive for NF- κ B in the EC compared to the control leg. Together, these data provide strong support for the activation of NF- κ B 3 h following EC.

Specific Aim #2: Determine both the cell type and subcellular localization of NF- κ B activation in human skeletal muscle following eccentric exercise.

Hypothesis #2: Immunohistochemistry (IHC), using antibodies against the p65 subunit of NF-kB and dystrophin will indicate the location (myonuclei vs. non-myonuclei) of expression and co-localization with nuclei in cross-sections of EC and control muscles.

Experimental Design: Immunofluorescent images of serial sections stained for the p65 subunit of NF-kB, dystrophin and nuclei were analyzed for co-localization of NF-kB to individual nuclei. The presence of the dystrophin stain provided information on whether the NF-kB positive nuclei were located inside the myofiber (myonuclei) or outside the myofiber (non-myonuclei). Follow-up analyses using antibodies against the p65 subunit of NF-kB and markers for various muscle resident cell types helped to identify the predominant cellular compartment of NF-kB activity.

Brief Results: Enumeration of NF-kB positive nuclei demonstrated that a significantly greater percentage of non-myonuclei with activated NF-kB than myonuclei. In fact, localization of NF-kB to myonuclei was a rare occurrence. Also noted was that a high number of these NF-kB positive non-myonuclei were positioned adjacent to or nearby what was perceived to be potential sites of microvasculature, as indicated by a tubular p65 staining pattern. This observation led to the hypothesis that NF-kB was being activated in cells associated with the microvasculature. Thus, muscle sections were co-stained with markers of vessel-associated pericyte cells and satellite cells. It was found that NF-kB localized predominately to cells that stained positive for markers of pericytes, indicating that pericytes, not satellite cells are the primary source of NF-kB activity in muscle following EC.

Specific Aims Study 3: The Effect of Pericyte Specific NF-kB Activation on Myogenic Response

The objectives of Study #1 and Study #2 were to use a paradigm of muscle damage in humans to further characterize early novel molecular changes that may be associated with muscle regeneration and adaptation. Preliminary observations from those studies suggested that activation of NF-kB in muscle residing pericytes may be an important event, although its significance within the framework of muscle regeneration (the overall dissertation objective) is yet unknown.

Recent attention has been paid to the role that NF-kB signaling plays in the myogenic response of developing and regenerating muscle (2, 5). In fact, there is ongoing debate as to whether it has primarily pro- or anti-myogenic effects, although there is strong emerging evidence to support the idea that the cellular compartmentalization of its activity is a key factor in its function (5). Similarly, there has been recent interest in the myogenic potential of muscle resident pericyte cells in normal and dystrophic skeletal muscle (6). Whether pericytes are myogenic and contribute to the muscle regenerative response following damage is unknown. Likewise, it is unknown if NF-kB signaling is important in the myogenic differentiation of pericytes following muscle damage or how NF-kB signaling in these cells may affect overall muscle regeneration by possible signaling relationships with other muscle progenitor cells (myoblasts/satellite cells). Thus, study #3 followed up on the functional relevance of pericyte specific NF-kB activation following muscle damage by employing an *in vitro* co-culture (pericyte/myoblast) model of myogenesis to understand how NF-kB dependent signaling

between these two cell types affects myogenic differentiation of myoblasts. In this model, I have both driven and inhibited NF-kB activity in pericyte cells and measured changes in myogenic and proliferative activity of C₂C₁₂ myoblasts. The specific aims of Study #3 were to:

Specific Aim #1: Determine the effect of altered NF-kB activation on the myogenic fate of primary pericyte cells in co-culture with C₂C₁₂ myoblasts and alone (monoculture) under differentiation conditions.

Hypothesis #1a: Enhanced NF-kB activation will result in decreased myotube formation in primary pericyte monocultures under differentiation conditions and a decreased contribution to myotube formation in co-culture with C₂C₁₂ myoblasts.

Hypothesis #1b: Diminished NF-kB activation will result in increased myotube formation in primary pericyte monocultures under differentiation conditions and an increased contribution to myotube formation in co-culture with C₂C₁₂ myoblasts.

Experimental Design: To test the effect of pericyte specific NF-kB activation on indices of myogenesis, I have transfected human placental-derived primary pericytes with expression vectors designed to enhance or diminish NF-kB activation. Transfected pericytes were then cultured under differentiation conditions in monoculture or in co-culture with differentiated C₂C₁₂ myotubes. IHC and Western blot were used to determine the level of pericyte differentiation in monoculture. For co-culture experiments, transfected pericyte nuclei were labeled with BRDU and pericytes were co-cultured with 4-day differentiated myotubes. Following 3 days in co-culture, IHC, using an antibody

against BRDU was used to identify and quantify the pericyte nuclei within C₂C₁₂ myotubes.

Brief Results: Despite published reports of spontaneous myogenic differentiation of placental-derived primary pericytes under reduced growth factor conditions (12), I was unable to observe evidence of myogenic differentiation, regardless of NF-kB activation status in monoculture. However, when pericytes were co-cultured with differentiated C₂C₁₂ myotubes, there was evidence of myogenic fusion to myotubes. Furthermore, as expected, diminished NF-kB activity significantly increased the relative number of myogenic pericytes compared to the control condition.

Specific Aim #2: Determine the effect of pericyte specific NF-kB activation on myoblast proliferation and myogenesis in an *in vitro* co-culture model.

Hypothesis 2a: Co-culture of myoblasts and primary pericytes, transfected with an expression vector to enhance NF-kB activation will result in increased myoblast proliferation and increased myotube formation.

Hypothesis 2a: Co-culture of myoblasts and primary pericytes, transfected with an expression vector to diminish NF-kB activation will result in decreased myoblast proliferation and decreased myotube formation.

Experimental Design: Human primary pericytes were transfected with the same expression constructs as in Aim #2 to alter NF-kB activity. Transfected pericytes were then co-cultured with C₂C₁₂ myoblasts under conditions to promote myoblast proliferation or under conditions to promote myoblast differentiation. Proliferation and differentiation of myoblasts were then measured under each of these conditions.

Brief Results: Pericyte-derived NF- κ B activity had a non-cell-autonomous effect on both differentiation and proliferation of co-culture myoblasts. When pericytes with enhanced NF- κ B activity were co-cultured with C₂C₁₂ myoblasts under differentiation conditions, there was a marked decrease in myotube formation and an increase in myoblast proliferation. Inhibition of NF- κ B activity in pericytes had no effect on either proliferation or differentiation of co-cultured myoblasts.

Summary

Skeletal muscle has a remarkable capacity to regenerate following injury. The complex molecular processes that drive muscle regeneration have received much recent attention, but are not fully understood. Although satellite cells are still thought to be the principal source of myogenic regenerative cells, we lack a complete understanding of the signals that drive satellite cells to differentiate. In this dissertation I present data to support a role for NF- κ B activation in muscle resident pericytes following a damaging exercise in humans (Chapter 3; Aims 1 and 2). Given that recent data have demonstrated that interstitially derived NF- κ B activity enhances myogenesis in developing muscle (12), it is likely to play a similar role during muscle regeneration following damage. Furthermore, evidence is emerging that non-satellite, muscle resident cells can contribute to the regenerative process via their local signaling influence, as well as their myogenic potential (13, 12). However, the relative contribution of these cells to muscle regeneration following injury has not yet been defined. In Chapter 4 (Aim 3), I define a potential role for these muscle constituents by showing that muscle resident pericytes are capable of cell- and non-cell-autonomous effects on myogenesis in an NF- κ B dependent

manner. The results of this dissertation have stretched our understanding of the molecular pathways involved in the muscle regenerative process and may advance our ability to treat muscle degenerative diseases such as muscular dystrophies.

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CHAPTER II

REVIEW OF LITERATURE

Introduction

Skeletal muscle is a plastic tissue, capable of repair and regeneration following damage. Its capacity to regenerate has been the focus of intense research over the past several decades, driven heavily by the need to understand the mechanisms and design treatments for muscle degenerative diseases such as Duchenne Muscular Dystrophy. Although extensive progress has been made in this area, we still know very little concerning the cellular interactions and signaling that result in a healthy muscle regenerative response. Furthermore, because of the genetic malleability of animal models and inherent difficulty of studying human skeletal muscle, the majority of work conducted to date on the mechanisms of muscle regeneration has been done in animals. Thus, there is a specific need for translational research in the area of muscle regeneration that focuses on relevant and safe human models of muscle damage and regeneration. This review of literature will therefore focus on what is currently known about skeletal muscle regeneration, its cellular and molecular mechanisms, and how the diverse cellular milieu that constitutes skeletal muscle interacts to effectively regenerate damaged muscle. Although much of what will be discussed has been developed in animal models, specific attention will be paid to the human literature. The review will conclude by discussing relevant models of muscle damage in animals and humans, including a review of eccentric contraction-induced damage, the model employed to study human muscle regenerative signaling in this dissertation.

Skeletal Muscle Development and Regeneration

Skeletal Muscle Development

Much of what we know about damage-induced muscle regeneration has been determined from studies of embryonic muscle development and cultured myoblasts. Skeletal muscle is derived from somites, originating on either side of the neural tube during vertebrate development (69). Entry of cells into the myogenic lineage is dependent on signals from surrounding tissue and ultimately, the expression of the myogenic regulatory factor (MRF) genes. MRFs are a group of basic helix loop helix transcription factors that act as master regulatory genes for skeletal muscle differentiation. They include the genes MyoD, Myf5, myogenin and MRF4. Genetic knockouts in mice have demonstrated the necessity for MyoD and Myf5 activity in the development of muscle (38). Mice null for either MyoD or Myf5 develop normally. However, mice null for both MyoD and Myf5 are born with no skeletal muscle, have no detectable levels of muscle specific mRNAs such as myosin heavy chain (MHC), and die shortly after birth from respiratory failure (38, 60). Thus, the expression of either MyoD or Myf5 is essential and sufficient for skeletal muscle development. Upon expression of the MRFs, MyoD and Myf5, proliferative cells withdraw from the cell cycle, commit to the myogenic lineage and express the “late” MRFs myogenin and MRF4, which are necessary for terminal differentiation into functional myofibers and ultimately, mature muscle (64). Figure 2.1 depicts the succession of transcriptional activation during muscle development.

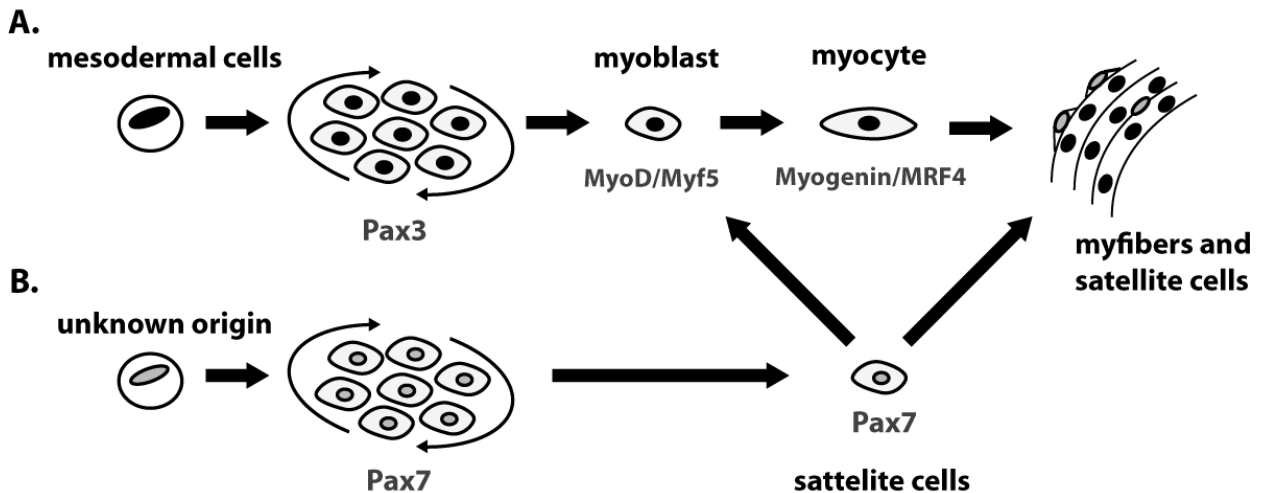


Figure 2.1: Origin and regulation of satellite cells in skeletal muscle development and regeneration. A) In muscle precursor cells, pax3 expression promotes expansion. Commitment of mesodermal cells to the myogenic lineage occurs upon expression of MyoD and Myf5. Given the correct signals, MyoD/Myf5 expressing cells upregulate myogenin and MRF4, which drive expression of muscle specific genes and promotes terminal differentiation. B) A distinct population of cells of unknown origin expand during early embryogenesis and express the transcription factor Pax7, which is required for satellite cell development and lineage determination. Pax7 expressing satellite cells can either upregulate MyoD/Myf5 and differentiate into skeletal muscle or can associate with the myofiber in a quiescent, undifferentiated state.

Skeletal Muscle Regeneration

Muscle regeneration after injury follows similar coordinated patterns of signaling and MRF expression as muscle development. It can be broadly characterized by a degenerative and subsequent regenerative phase. Mechanical- or chemical-induced damage compromises the sarcolemma and can be recognized by the presence of muscle specific proteins such as creatine kinase in the blood serum. In response to sarcolemmal damage, the initial degenerative phase is thought to first involve the loss of calcium homeostasis, thereby activating calcium dependent caspases, which cleave and assist in the degradation of damaged myofibrillar proteins (6). This process is followed closely by the activation of specific waves of phagocytic inflammatory cells. It has been hypothesized that chemotactic factors released by the damaged muscle activate residing

and circulating inflammatory cells to initiate the inflammatory response, although there is little direct evidence to support this hypothesis. Interleukin-8 (IL-8) is a chemokine that is increased in blood serum very early (1-3h) following damaging eccentric exercise, but not concentric exercise, making it a likely candidate to signal the initial inflammatory response following muscle damage (1). The degenerative period is closely followed and somewhat overlapped by a regenerative phase, characterized by the expansion and subsequent fusion of myogenic progenitor cells to the damaged myofiber, or to one another for *de novo* muscle fiber formation (13).

Muscle Progenitor Cell Populations

Satellite Cells

Muscle regeneration following damage is dependent on a pool of undifferentiated muscle progenitor cells that are capable of dividing, differentiating and fusing to damaged fibers. During development, a subset of Pax7 expressing cells, coined satellite cells, fail to differentiate and remain associated with the myofiber in a quiescent state (Figure 2.1B). Satellite cells are undifferentiated and similar in appearance to both embryonic and fetal myoblasts but express distinct genes during development and thus appear to be fundamentally unique to these cell populations (26). They are distinguished from other cell types and from myonuclei by their location within the basal lamina surrounding myofibers, their high nuclear to cytoplasmic ratio and reduced organelle content. Satellite cells play an integral role in regeneration of skeletal muscle following damage. In a coordinated and well-characterized fashion, satellite cells become activated, enter a

proliferative phase and then fuse to areas of damaged muscle (Figure 2.2A). The necessity of satellite cells in regenerative muscle is well-illustrated by the inability of *pax7* null mice, which lack satellite cells, to effectively regenerate muscle following damage (41). As in the developmental stages, the MRFs MyoD, Myf5 and myogenin play pivotal roles in the activation and terminal differentiation of satellite cells. Using transcriptome analysis, Zhao and Hoffman (76) have perhaps provided the most detailed description of the transcriptional profile following muscle injury during 27 regenerative periods. Their data indicate a pattern of gene expression for the MRFs and MRF associated genes that is similar to muscle development and begins as early as 2-3 hours post-injury in mice. In fact, Cooper et al. (17) have shown in a mouse model that both MyoD and Myf5 are activated in satellite cells as early as 3 hours post-damage. Furthermore, they report that at 12 hours post-damage, the activation of MyoD and Myf5 are no higher than at 3 hours, indicating the importance of early activation. Figure 2.2B details the time course of the stages in regenerative muscle.

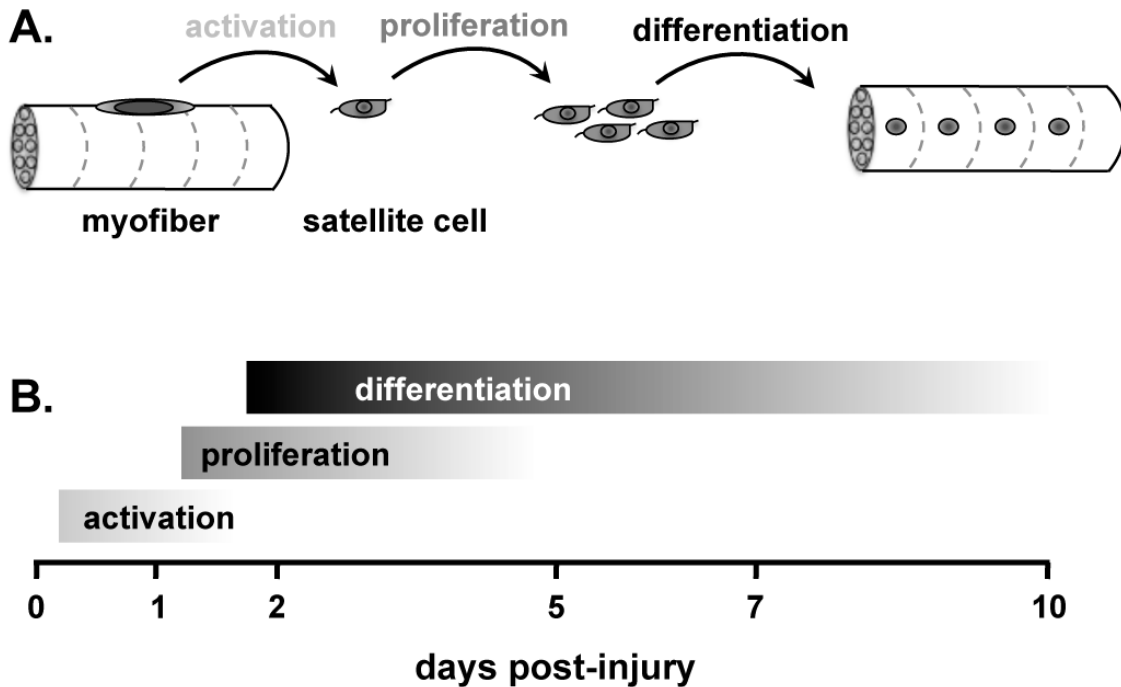


Figure 2.2: Coordinated muscle regeneration following damage. A) Following muscle damage quiescent satellite cells are activated, proliferate, migrate to areas of damage and differentiate to repair damaged muscle. B) A time course of satellite cell activation and differentiation following damage.

Other Muscle Progenitor Populations

In addition to the demonstrated regenerative capacity of satellite cells, it is now becoming clear that other cell populations are likely involved in limited muscle regeneration. Both muscle and non-muscle derived cells have been shown to possess myogenic potential. For example, a pioneering study by Ferrari *et al.* (27) showed that bone marrow derived cells can be recruited and differentiated into myofibers following injury. Other groups have similarly shown that marrow cells have myogenic potential and express MRF genes at high levels when delivered or co-cultured with murine muscle (18, 37). More recently, blood vessel derived pericytes isolated from human biopsy samples showed

rates of myogenic differentiation similar to that of satellite cells, both when they were co-cultured with C₂C₁₂ myoblasts or cultured alone under low growth factor conditions (24). This finding underscores a potential role for these cells in injury-induced muscle regeneration. In support of this assertion, Dellavalle *et al.* (24) further demonstrated that muscle-derived pericytes, when transplanted into dystrophic mice, gave rise to new dystrophin expressing fibers. Because pericytes isolated from skeletal muscle are likely to be contaminated with satellite cells, studies using muscle-derived pericyte cells have been criticized. In response to this criticism, Kirillova *et al.* (40) used a pericyte population isolated from bovine retinal tissue to test the myogenic capability of this cell type. Their data suggest that retina-derived pericyte cells can spontaneously fuse with preexisting myotubes in co-culture. Most recently, pericytes derived from human placental tissue have been shown to have myogenic potential and significantly rescue dystrophin expression in an *mdx* mouse model of dystrophy (57).

Thus, there is evidence to support the notion that muscle and non-muscle residing cell populations, distinct from satellite cells, are capable of, and likely contributing to myogenesis following injury. However, our understanding of the signaling pathways and mechanisms that govern that myogenic commitment *in vivo* is lacking. Furthermore, the functional interactions and signaling between these and other muscle residing cell types following muscle injury are relatively unknown. Future studies that employ the isolation of primary myogenic cells from regenerating muscle via flow cytometry may perhaps help to reveal some of these important mechanisms.

Inflammatory and Muscle-Residing Cell Signaling

The composition of skeletal muscle can be most accurately described as a heterogeneous mix of multi- and mono-nucleate cell types. In addition to the multi-nucleate myofibers, several mono-nucleate cells have been identified to both reside in and infiltrate skeletal muscle following injury. While we still know relatively little about the functional interactions between these distinct cell types, it is clear that they play an integral role in the acute response and subsequent regenerative response following damage.

Inflammatory Cells

Following acute injury, distinct populations of inflammatory cells infiltrate muscle in a predictable pattern. Between 2 and 24 hours post-injury, neutrophils respond first followed by a wave of phagocytic macrophages at 24-48 hours post-injury. This response is closely followed by the invasion of a second wave of non-phagocytic macrophages between 48 and 72 hours post-injury (72). Interestingly, the timing of these inflammatory cell population waves correlates well to the expression patterns of the myogenic transcription factors (MyoD, Myf5 and myogenin) in MPCs; most notably, the satellite cells. Therefore, it has been hypothesized that chemokine signaling between infiltrating inflammatory cells and satellite cells plays a key role in the initiation and progression of muscle regeneration. For example, in multiple models of muscle injury in rodents, MyoD expression in satellite cells has been shown to peak at 48 hours post-injury, coincident with the decline in phagocytic macrophages and the increase in the non-phagocytic macrophages (43, 52). The effect of inflammatory cell signaling on myogenesis is further

illustrated *in vitro* by the co-culture of specific inflammatory cell populations with C₂C₁₂ myoblasts. When co-cultured with cells of the macrophage lineage, C₂C₁₂ myoblast proliferation is enhanced and associated with more MyoD positive nuclei (49). More recently, several studies have focused on the specific inflammatory cell derived chemokines responsible for driving satellite cell proliferation and myogenesis. Some of the more well-studied chemokine proteins include monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), macrophage inflammatory protein-1 β (MIP-1 β) and leukemia inhibitory factor (LIF), all of which have been shown to affect satellite cell proliferation and myogenesis *in vitro* and *in vivo* (73, 74). Furthermore, knockout mouse models for several of these chemokines have shown compromised muscle regeneration (16, 65). Thus, evidence is accumulating to support a central role for inflammatory cell signaling in muscle regeneration following injury.

Other Muscle-Residing Cells

In addition to infiltrating inflammatory cells, signaling by other muscle residing cell types can influence muscle regeneration. A recent investigation by Dahlman *et al.* (22) has demonstrated that stromal fibroblasts can promote myogenesis in developing neonatal muscle tissue via nuclear factor-kappaB (NF-kB) dependent nitric oxide synthase (iNOS) signaling. Given the parallels between myogenesis during development and following injury, this represents a possible mechanism for enhanced myogenesis following injury. A small population of non-phagocytic macrophages has also been described to reside in skeletal muscle (51). However, there are limited data available on the functional relationship between these residing macrophages and other cells involved

in muscle regeneration. It would be logical to assume, given their immediate proximity to the muscle fibers, that they may be involved in the signaling of an early inflammatory response to injury. Overall, the contributions of non-satellite cells to the muscle regenerative process following injury is not very well characterized or understood.

Myofibers

Given that compromise of the membrane of individual myofibers is observed following muscle damage (53), it has been hypothesized that factors released from damaged myofibers result in activation of MPCs. Several growth factors that are known to be produced and secreted by myofibers including HGF, IGF-1 and FGF have been shown to activate satellite cells and suggested to be important in signaling their activation following damage (2, 71). However, the ultimate source of these trophic factors following damage remains uncertain. Likewise, members of the interleukin (IL) family of proteins have been suggested to be released from damaged myofibers and contribute to satellite cell activation. Recent work by McKay *et al.* (50) have shown that IL-6 plays a key role in satellite cell activation and proliferation in human muscle, 4 hours following ECC-induced damage. Other studies have measured serum concentrations of IL-8 following both concentric and eccentric exercises (11, 55). Interestingly, IL-8 serum concentration is increased only following exercise with an eccentric component (e.g. running), leading some to speculate that it may signal be released from damaged myofibers to signal the inflammatory response. Although, IL-8 mRNA levels have been shown to increase following eccentric exercise (8), the ultimate cellular source (i.e. myofiber, satellite cell etc.) of IL-8 has not been determined. However, of particular note,

is that both IL-6 and IL-8, in addition to several of the aforementioned growth factors are regulated at the mRNA transcript level by activation of the transcription factor Nuclear Factor kappa-B (NF- κ B).

NF- κ B Signaling in Muscle Regeneration and Myogenesis

NF- κ B Signaling

Nuclear Factor kappa-B (NF- κ B) belongs to a family of transcription factors consisting of 5 proteins (p65, c-Rel, RelB, p50 and p52). NF- κ B is maintained as a heterodimeric protein complex (most commonly p50/p65) that resides in the cytosol of cells in its inactive form, held there by its inhibitor protein I κ B. It is activated by a well-known mechanism involving the phosphorylation of the IK-kinase (IKK) complex. IKK in turn phosphorylates I κ B, resulting in its ubiquitin-dependent degradation and subsequent release of NF- κ B. Upon its release from I κ B, NF- κ B translocates to the nucleus where it activates transcription of a host of dependent genes.

The degradation of I κ B can be mediated by two distinct signaling pathways; the classical and alternative pathways. The classical pathway generally refers to the activation of the p50/p65 heterodimer. Although there is some evidence that the alternative pathway (RelB/p100 heterodimer) is important in muscle function and myogenesis (5), this review will deal primarily with the classical pathway, as the majority of research has focused here. A schematic of the classical NF- κ B signaling pathway is shown in Figure 2.3.

Activation of NF- κ B is primarily stimulated in response to inflammatory cytokine activity including interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) (62), in addition to growth factors such as insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF) (31). NF- κ B, in addition to functioning as a well-studied pro-inflammatory transcription factor, is also involved in the transcriptional regulation of genes associated with developmental processes (22), cell growth and proliferation (7), and survival (3). However, this dissertation will focus on its emerging role during skeletal muscle differentiation and regeneration.

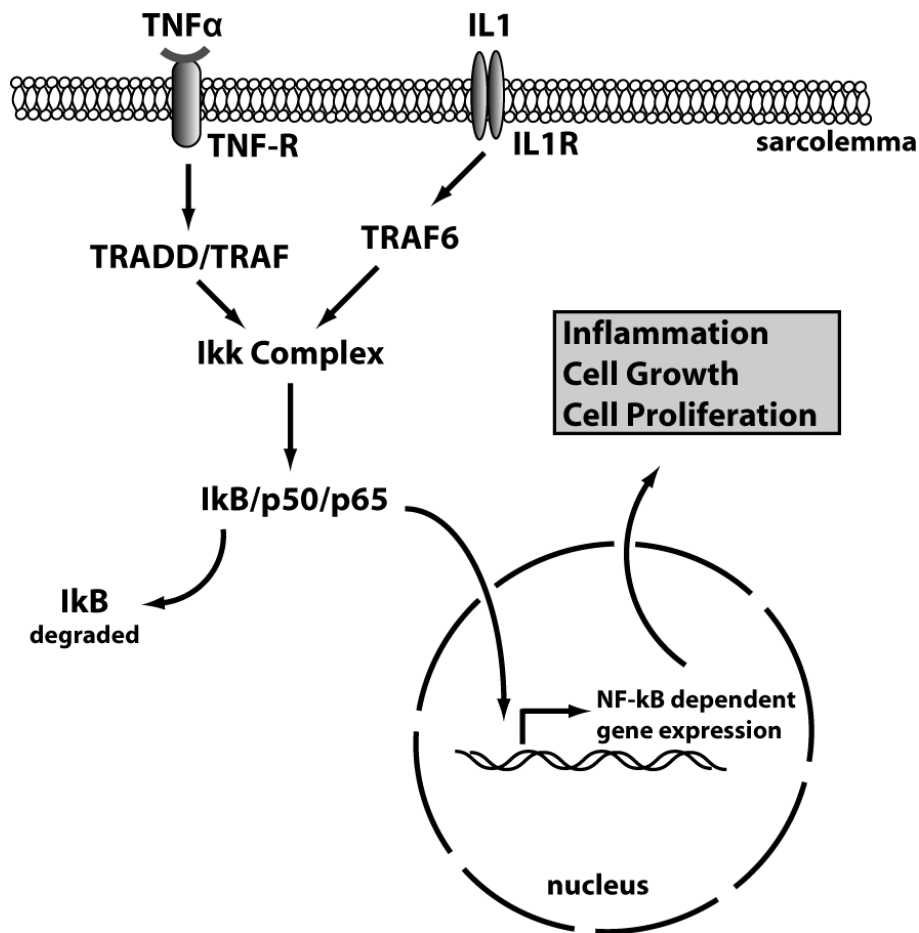


Figure 2.3: Classical NF- κ B signaling pathway

NF- κ B and Muscle Regeneration

In muscle, a consensus on the effect of NF- κ B on developing and regenerating muscle has not been made. However, the majority of evidence supports that the NF- κ B signaling pathway acts as an inhibitor of myogenesis. Early reports in cultured myoblasts have associated differentiation with a decline in NF- κ B activation (45). More direct evidence indicates that NF- κ B activation can both promote the degradation of MyoD and suppress the expression of myofibrillar genes (42, 68). In mice, the inhibition of NF- κ B activation has also been shown to enhance satellite cell activation and facilitate muscle regeneration following muscle damage (54). In contrast, *in vitro* studies have reported that NF- κ B is required for differentiation (4, 10). This pro-myogenic effect has been further demonstrated *in vivo* by Dahlman *et al.* (22), who observed high NF- κ B activity in the first two weeks of mouse neonatal development, a period of growth associated with high levels of myogenesis. Importantly, their subsequent analysis of the localization of NF- κ B activity showed that the increases in activity originated from muscle-residing stromal fibroblasts and not the muscle fibers. Furthermore, they showed that NF- κ B activity in stromal fibroblasts promoted myoblast fusion via nitric oxide synthase (iNOS) signaling. This study provided novel and important insight into the role that cellular compartmentalization plays in the molecular signaling of muscle. Future work should now be directed towards elucidating this same effect in regenerating adult skeletal muscle.

Interestingly, there are few reports in humans on the function of NF- κ B signaling in the regenerative response that follows muscle damage. This is somewhat surprising in light of the fact that muscle damage results in a predictable and well-characterized inflammatory and oxidative stress response (48, 72), both of which are associated with activation of NF- κ B (35). The few studies that have measured NF- κ B activation following what might be considered a damaging exercise have reported either a decrease or no change in its activity (12, 25). For example, Durham *et al.* (25) reported decreased NF- κ B DNA binding activity via electrophoretic mobility shift assay (EMSA) immediately following a lower body resistance exercise (eccentric and concentric work). In contrast to the previous human work, studies in rodents have demonstrated a clear pattern of NF- κ B activation following muscle contraction and/or an acute bout of exercise (32, 36). Furthermore, treadmill running in rats, which has a strong eccentric, damaging component, has been shown to increase NF- κ B binding activity, enhance I κ k phosphorylation and increase IL-6 mRNA expression, all of which suggest activation of NF- κ B (36, 67). Thus, given its well-established role in skeletal muscle myogenesis *in vitro* and in rodents, future studies should focus on a function for NF- κ B in the regenerative response of human muscle following damage.

Other Signaling Pathways Associated with Muscle Regeneration

PI3k/Akt

The maintenance of skeletal muscle mass, in addition to being a function of balanced protein synthesis and degradation, is also regulated by *de novo* myogenesis. For example,

a hallmark of both sarcopenia (age-related loss of muscle mass) and cachexia (disease-related loss of muscle mass) is a progressive incapacity of satellite and other progenitor cells to repair damaged or degenerative muscle (58, 63). Myogenesis is regulated in satellite cells by the transcriptional activation of a group of genes known collectively as the myogenic regulatory factors (MRFs). They include the genes MyoD, Myf5, myogenin and MRF4. In response to various stimuli, for example, damage caused by strenuous exercise, a subset of muscle satellite cells become activated and begin to express MyoD and Myf5. These “activated” cells, known as myoblasts, become committed to the myogenic lineage and soon thereafter begin expressing myogenin and MRF4. Upon expression of these “late” MRF genes, myoblasts migrate to areas of damaged muscle and fuse to the damaged muscle fibers. A schematic of this process is provided in Figure 2.2.

In addition to its well-described regulatory role in the maintenance of mass post-mitotic muscle mass, Akt has been implicated to function in myoblasts to regulate myogenesis. Early work by Fujio *et al.* (28) showed that Akt activity promotes both myoblast cell cycle withdrawal and cell survival. Subsequent work demonstrated that this regulation occurs through phosphorylation of the FOXO1a transcription factor that, when manipulated to disallow Akt phosphorylation, remained in the nucleus and prevented myoblast differentiation (33). More recent studies have shown that myoblasts lacking Akt have a marked decrease in the production of muscle-specific proteins and a reduced capacity to differentiate into multinucleated myotubes (59). Further support for the pro-myogenic effects of Akt activation has been provided by Kim *et al.* (39), who used

overexpression of a constitutively active Akt to promote muscle regeneration and improve measures of muscle function in a dystrophic mouse model. Thus, although the developing consensus supports the notion that Akt acts as a pro-myogenic regulator, the mechanisms of its action are still unclear. For example, many have speculated that Akt promotes myogenesis via the regulation of the MRFs MyoD and myogenin; however, there are no data to support this hypothesis to date.

p38 Mitogen Activated Protein Kinase (p38 MAPK)

Activation of p38 MAPK is considered to be an essential and early event in myogenesis *in vitro* (9) and during early embryonic development (23). It is generally regarded as a molecular switch for the activation of myogenesis through multiple mechanisms. One of its more well-documented mechanisms of action involves the phosphorylation and activation of chromatin remodeling complexes, which facilitate MyoD and myocyte enhancer factor (MEF)-2 binding to the myogenin promoter (66). p38 MAPK activation has also been shown to enhance MyoD nuclear translocation and muscle specific gene transcription (46). Despite its well-established role as a pro-myogenesis factor during embryogenesis and *in vitro*, the role of p38 MAPK activity following injury is less clear. For example, myogenic regulatory factor-4 (MRF-4), a member of the MRF family of transcription factors, is de-activated following phosphorylation by p38 MAPK, leading to transcriptional repression of myogenic genes (70). Moreover, p38 MAPK deficient mice display normal muscle regeneration following chemically induced muscle injury (61).

Overall, there is strong evidence to support the pro-myogenic requirement of p38 MAPK activation in developing muscle. However, in regenerating adult skeletal muscle, the limited data available to date are inconclusive. Clearly, more work is needed to adequately define a functional requirement for p38 MAPK in muscle regeneration. Future studies on the effect of p38 MAPK activation on the myogenic differentiation of non-muscle derived cells would also be important, as they may perhaps lead to potential therapeutic targets for muscle degenerative diseases.

Animal and Human Models of Muscle Damage

To adequately study the mechanisms of skeletal muscle regeneration, an experimental model of muscle injury is necessary. Several models of muscle injury have been developed in animals, humans and in isolated single muscle fibers, each with distinct strengths and weaknesses.

Animal Models

The injection of myotoxins into animal muscle tissue has been a mainstay for the mechanistic study of muscle regeneration for several decades (21, 30). Employed in large part due to its reproducibility and convenience, localized myotoxin injection results in membrane disruption and lysis of various cells. Within 1 to 3 days, the muscle is infiltrated with mononuclear inflammatory cells and *de novo* myotube formation and myogenesis is generally observed within 5 to 6 days post injection. Although effective at producing local muscle injury, the unknown effects of the toxin on other cell types,

including satellite cells have called into question its physiologic relevance. Consequently, other models of muscle damage in animals have been developed. Some of these include direct infliction of damage by crushing or freezing. Although not as predictable and controlled as myotoxin injection, these methods have been shown to generally result in muscle injury and subsequent regeneration (44). Alternatively, though more technically difficult, high intensity exercise, specifically exercise with an eccentric component has also been shown to be effective at inducing muscle damage in animals, albeit at a much lower level than the aforementioned methods (34).

Human Models

Due primarily to ethical considerations, experimental paradigms of muscle injury in humans are much more limited. However, one approach that has exploited a common mechanism for normal and transient muscle damage in humans is the induction of muscle injury via eccentric exercise. It has been very well documented that eccentric contractions do indeed produce muscle damage and subsequent adaptation (15, 29). Damage is evident at the ultra-structural level by disrupted sarcomere structure (75), at the cellular level by loss of membrane integrity and necrosis (19, 53), and at the functional level by decreases in strength and increases in soreness (15). Furthermore, the recovery from eccentric-induced damage involves a significant regenerative response wherein satellite cells are activated and incorporated into damaged fibers (19, 20, 56). Most recently, O'Reilly *et al.* (56) reported an increase in satellite cell number as early as 24h post-eccentric exercise of the knee extensors in humans, suggesting that eccentric exercise-induced damage is

sufficient to initiate a regenerative response and represents an apt model for the study of regenerative pathways in humans.

To date, multiple variations of the eccentric exercise paradigm have been developed. Of these, perhaps the most frequently used is injury of the elbow flexors. Our laboratory has also pioneered the use of a modified preacher curl bench, wherein an extended lever arm provides the researcher with the ability to force the arm into extension as the study participant maximally resists with the elbow flexors. These methods have been repeatedly shown to result in muscle damage as indicated by prolonged strength loss, soreness and increased serum CK and myoglobin (indirect markers of muscle damage) (15). The knee flexors have also been used frequently as the target of muscle damage studies, likely because they are larger and easier to extract tissue from via a biopsy. Similarly, eccentric contraction-induced damage of the knee flexors has been shown to produce robust and repeatable changes in the direct (sarcomere disruption) and indirect markers of muscle damage (14, 47, 56). The following investigations presented in this dissertation on the molecular changes following muscle damage have used this model to effectively induce transient muscle damage. Studies employing models such as these will be important in determining the underlying mechanisms and molecular signaling responsible for the muscle regenerative response in humans to damage.

Conclusion

Muscle regeneration has, for many decades, been thought to occur exclusively via the action of muscle-resident satellite cells (SC). However, evidence has recently emerged that non-satellite muscle- and non-muscle- resident cells may play a role in the regenerative response and may, in fact, contribute both directly (myogenic differentiation) and indirectly (signal myogenic differentiation) to *in vivo* muscle repair. However, the extent to which this occurs in human muscle is unknown, as are the signaling pathways responsible for driving their myogenic differentiation. In the following studies, I have used an eccentric contraction model of muscle damage (Chapter 3) and an *in vitro* model of myogenesis (Chapter 4) to show that micro-vessel associated pericyte cells (both muscle and non-muscle derived) may influence muscle differentiation following damage via activation of the NF-kB signaling pathway. The results of these studies have provided insight into the mechanisms that may drive myogenic commitment and will have implications for potential molecular targets to enhance muscle regenerative action in satellite and non-satellite muscle progenitor cells.

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CHAPTER III

ACTIVATION OF NUCLEAR FACTOR-KappaB FOLLOWING MUSCLE ECCENTRIC CONTRACTIONS IN HUMANS IS LOCALIZED PRIMARILY TO SKELETAL MUSCLE RESIDING PERICYTES

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Introduction

Skeletal muscle has a dynamic capacity to adapt following changes in functional demand or damage. Its adaptive response, which is dependant on the activation and regenerative action of muscle progenitor cells (1), is essential for the maintenance of muscle mass and, when dysregulated as a result of disease or aging (2, 3), can lead to significant losses in functional capacity and increased mortality (4). The activation of muscle progenitor cells, most notably satellite cells, likely involves complex signaling between the myofibers, their interstitial environment and other systemic factors. Significant advances in the understanding of these signaling relationships and mechanisms have been made in recent years. For example, smooth muscle and fibroblastic cell derived signaling was shown to influence activation and self-renewal of satellite cells (5). Likewise, growth factors such as fibroblast growth factor (FGF) (6), insulin-like growth factor 1 (IGF-1) (7) and hepatocyte growth factor (HGF) (8) have been shown to influence satellite cell activation and muscle regeneration, although their cellular origins during *in vivo* muscle regeneration are largely unknown. Despite these advances, there exists a significant gap in our understanding of these relationships in human skeletal muscle, as the majority of these studies have been done in rodent or cell models, which may not completely recapitulate the regenerative events in human muscle (9). Thus, the further

characterization of the molecular mechanisms that drive the regenerative and adaptive response in human muscle is imperative to enhance our current modes of therapy in diseased or aged muscle.

The performance of eccentric contractions (EC) has been shown to safely and effectively induce transient muscle injury that results in a well-characterized regenerative response involving the activation and proliferation of muscle satellite cells (10-12). We, and others have used this model in humans to evaluate changes in global gene expression and have identified changes in the transcriptional activity of genes associated with inflammation, protein synthesis, extracellular matrix remodeling and myogenesis (13-16). However, due perhaps to the invasive nature of extracting muscle tissue from humans or the associated cost of commercial microarray platforms, subject sample size in these studies has been small. For example, Chen et al. (13) report gene expression changes via microarray analysis following EC in 3 male subjects. Likewise, others have data using similar methods in 3 (15), 6 (14) and 4 (16) subjects. Thus, while these studies have made significant contributions in ascertaining some of the more highly dysregulated genes following EC (13, 15, 16), their small sample sizes have precluded a network analysis, wherein detection of moderate changes (not likely to be seen with a small sample) in multiple or groups of genes enhances the ability to identify important biological pathways involved in muscle adaptation and regeneration after EC.

Therefore, the present study was undertaken with the primary objective of further characterizing the early molecular response to EC in humans by: 1) conducting a well

powered, genome wide screen and systems level analysis at the transcriptional level and 2) using these data to derive testable hypotheses for follow up analyses. We expected that our approach would provide a novel transcriptomic profile highlighting potential pathways and networks that may govern early changes suggestive of muscle regeneration and adaptation and that our follow-up analyses would confirm these findings.

In the following study we provide support for the transcriptional activation of both previously reported and novel genes following EC in humans. More importantly, our follow up analyses offer compelling evidence for the activation of the NF- κ B transcription factor complex in human skeletal muscle shortly following EC and the localization of its activity primarily to muscle residing pericyte cells.

Methodology

Subjects: Thirty-five healthy male subjects (20.9 ± 0.5 yrs, 178.9 ± 1.2 cm and 80.6 ± 2.9 kg) volunteered for this study and had muscle biopsies sampled from the control (non-EC) and EC legs. All subjects signed a written informed consent document approved by the University of Massachusetts Amherst Institutional Review Board. All study procedures conformed to the standards outlined in the *Declaration of Helsinki*. Subjects had no prior history of musculoskeletal injury of the lower extremity and were willing to refrain from participating in new physical activities or taking oral or topical analgesics for the duration of the study. Subjects were excluded if they had been involved in a

strength-training program in the past 6 months or routinely lifted heavy objects or engaged in activity with a heavy eccentric component.

Study Design: The study consisted of 7 visits over the course of 1 week. Subjects reported to the laboratory for their first visit fasted and had their blood drawn for baseline creatine kinase (CK) activity measurements. The blood draw was followed by an orientation to the Biodex System 4 dynamometer (Biodex Medical Systems, Shirley, NY) and subsequent baseline strength tests on a randomized (dominant vs. non-dominant) leg. The following day (24 h after visit 1), subjects once again reported to the laboratory in a fasted state (12 h). Upon arrival they were provided a standardized breakfast consisting of 400 kcal (approximately 55% carbohydrate, 30% fat and 15% protein). Subjects then performed a baseline soreness/pain assessment using a visual analog scale (VAS). This was followed by a muscle strength assessment and the eccentric contraction protocol. Muscle strength was assessed again immediately following the contractions. Three hours after the completion of the contraction regimen, a muscle biopsy was taken from the vastus lateralis of both legs. The non-eccentrically contracted leg was designated the control; the eccentrically contracted leg was designated as EC. Muscle strength and soreness were then assessed every 24 ± 2 h for 120 h on the EC leg; CK levels were also measured every 24 h during the same period.

Contraction Protocol: We used a contraction protocol similar to that developed and used previously by others to induce muscle damage (16). Subjects completed 100 maximal eccentric contractions using a Biodex isokinetic dynamometer. Subjects were seated in

the dynamometer and a randomized (dominant vs. non-dominant) leg was strapped to the lever arm. Subjects were first asked to extend the lever arm as far as possible. This position was designated as 0 degrees of flexion. From this point of reference, the dynamometer limits were set to exercise the subject through a range of motion of 35 degrees of knee flexion to the subject's maximal flexion angle. Total range of motion averaged about seventy-five degrees. The protocol consisted of 10 sets of 10 repetitions with 10 s rests between repetitions and 1 min rests between sets. For each repetition, subjects were instructed to maximally extend or "kick" as the dynamometer moved from 30 degrees of knee flexion to the subject's maximal flexion angle at 30 degrees per second. Following each repetition, the dynamometer returned the leg to 30 degrees of knee flexion.

Muscle Biopsy: Percutaneous needle biopsies of both the EC and non-EC vastus lateralis muscle were obtained 3 h following the eccentric exercise. Under local anesthesia (Lidocaine), a small incision was made into the skin and fascia, and the biopsy needle was inserted into the muscle. A small core of tissue (~100mg) was withdrawn and was immediately frozen in liquid nitrogen. The tissue was then stored at -80°C until it was used for analyses.

Knee Extension Strength Testing: A measure of maximal isometric strength of the knee extensor muscles was assessed on the Biodex dynamometer at an angle of 70 degrees of flexion. Subjects completed three 3 s isometric contractions with a 1 min rest between

trials. Peak isometric torque values were defined as the average of the highest attainable values for each of the 3 trials.

Muscle Soreness: Soreness was evaluated using a visual analog scale (VAS); a 100mm line with 0mm indicating “no pain” and 100mm indicating “unbearable pain.” Subjects marked the scale with a single vertical line following two hip/knee flexion and extensions (sitting and rising from a chair). The distance from the left end of the scale to the mark was taken as the soreness level. All subjects provided a pre-EC VAS evaluation. If the baseline VAS score was determined to be greater than 10mm, the subject was asked to return two or more days later to allow any soreness to dissipate.

Total RNA Isolation and Microarray Hybridization: Total RNA from EC and control samples of 35 subjects were extracted using the standard TRIzol (Invitrogen, Carlsbad, CA) method. mRNA was then isolated using the Oligotex mRNA Midi kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Double stranded cDNA was created using the Ovation Pico WTA system (NuGEN, San Carlos, CA) according to the manufacturer’s instructions. Cy3 labeling and hybridization on to an Agilent Whole Genome Microarray (Agilent, Santa Clara, CA) were done at Gene Logic Inc. (Gaithersburg, MD) according to the manufacturer’s specifications.

qRT-PCR Analysis: 100ng of total RNA from each sample was reverse transcribed using a cloned murine leukemia virus reverse transcriptase (Fermentas, Glen Burnie, MD) according to the manufacturer’s instructions. The relative levels of *Ikba*, *ICAM1*,

TNFRSF1A and *CEBPD* were measured by quantitative real-time (qRT) PCR. ABgene Absolute qPCR SYBR Green Master Mix (ABgene, Surrey, UK) with ROX dye was used for all PCR protocols. qRT-PCR reactions were performed in 96-well plates with all cDNA samples from both EC and control legs run in triplicate for each gene of interest along with no template controls. The average Ct (cycle threshold) value for triplicate samples was used for data analysis. Ct values were directly related to fluorescence of the respective SYBR-green probe after 40 cycles of amplification on a MX3000p Real-Time PCR System (Stratagene, La Jolla, CA). At the end of each reaction, a melting curve analysis was run to identify possible primer dimers. The results of the melting curve analysis was confirmed, and appropriate product size determined via 2% Agarose gel electrophoresis with ethidium bromide staining.

Table 3.1: qRT-PCR primers

Gene Name	Forward Primer	Reverse Primer
<i>CEBPD</i>	5' - AGTTTCTTGGGACATAGGAGCGCA - 3'	5' - GTACCTTAGCTGCATCAACAGGAG - 3'
<i>ICAM1</i>	5' - AAGCCAAGAGGAAGGAGCAAGACT - 3'	5' - AGGCTTGGTAAACATCTCCAGGCA - 3'
<i>IkBa</i>	5' - TGGCCTTCCTCAACTTCCAGAACA - 3'	5' - CTCAGCAATTTCTGGCTGGTTGGT - 3'
<i>B2M</i>	5' - TGTCTGGGTTTCATCCATCCGACA - 3'	5' - TCACACGGCAGGCATACTCATCTT - 3'

Differences in relative gene expression were determined by the $\Delta\Delta\text{CT}$ method. Values were normalized to expression of Beta-2-Microglobulin (*B2M*), an internal control, previously validated for eccentric exercise studies (18). Forward and reverse primers (Integrated DNA Technologies, Coralville, IA) of all genes of interest were tested for

efficiency. All primers had efficiencies between 95 and 105% and thus were deemed appropriate to assess relative expression via the $\Delta\Delta\text{CT}$ method. Primer sequences are presented in Table 1.

ELISA-based NF- κ B Activation Assay: Due to limited tissue, NF- κ B activation analyses (ELISA and immunohistochemistry) were performed on EC and control biopsy samples of a subset of 15 subjects. Nuclear extracts were prepared by homogenizing tissue samples in a low salt lysis buffer (10mM HEPES pH 7.6, 10mM KCL, 1.5mM MgCl₂, 0.1mM EDTA, 0.1 mM DTT, 0.5 M PMSF, 50 μ l protease inhibitor cocktail and 0.5mg/ml benzamidine). Homogenized tissue was then subjected to 2 cycles of freeze/thaw using an ethanol/dry ice freeze bath and a 37°C water bath. Samples were then vortexed and centrifuged at 3000 RPM for 3 min at 4°C. The supernatants (cytosolic protein) were then pipetted off and the nuclear pellet was re-suspended in high salt buffer (20mM HEPES pH 7.6, 420mM NaCL, 1mM EDTA, 25% glycerol, 1 mM DTT and 5 μ l protease inhibitor cocktail). The samples were then incubated on ice for 30 min after which they were centrifuged at 13,000 RPM for 5 min at 4°C. The supernatant, containing the nuclear protein fraction was pipetted off and stored and quantified. A standard BCA assay (Pierce, Rockford, IL) was used to quantify nuclear protein. NF- κ B activation was determined using nuclear extracts and an ELISA-based TransAM NF- κ B p65 assay kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 5 μ g of nuclear extract were added to wells coated with a consensus binding sequence for NF- κ B (5'-GGGACTTTCC-3') and incubated for 1h at room temperature. Wells were then washed and a primary antibody directed at the p65 subunit was added

and left to incubate for 1h. This was followed by treatment of all wells with a secondary antibody conjugated to horseradish peroxidase (HRP). A subsequent colorimetric reaction was initiated with the addition of a developing solution for 5 min followed by the application of a stop solution. The absorbance of the plate was then read at 450nm on a multi-well microplate reader (FLUOstar Optima, BMG LABTECH). Wild type and mutated consensus oligonucleotides were used as competitors for NF- κ B binding to ensure specificity of the reaction as per the manufacturer's instructions. All samples were run in duplicate and the average value was used for data analysis.

Immunohistochemistry (IHC): 10 μ m cross sections of muscle biopsy tissue samples from EC and control legs of the same 15 subjects were generated on a MICROM HM 505E cryostat at -25°C (Richard Allan Scientific, Kalamazoo, MI). Samples were mounted to Superfrost slides (Fisher Scientific) and air-dried for 30 min. Sections were re-hydrated with 1XPBS with 0.01% N₂Na for 5 min and blocked in 5% BSA (Invitrogen, Carlsbad, CA) for 15 min at room temperature. Sections were then incubated in the appropriate primary antibodies, diluted 1:100 in 5% BSA for 1h at 37°C. Following incubation, sections were washed 3 times in 1X PBS for 5 min and blocked again in 5% BSA. Sections were then incubated in appropriate secondary antibodies and 49,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) or Topro3 (for confocal microscopy) (Invitrogen, USA) for 30 min at 37°C, washed in PBS and mounted. Stained slides were imaged on either a Nikon model TE-2000 inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI), or a Zeiss 510 META confocal laser scanning microscope. The following primary antibodies were used: NF- κ B-p65

(polyclonal, 1:100; Abcam, Cambridge, MA), NG2 (monoclonal, 1:100; Abcam, Cambridge, MA), dystrophin (monoclonal, Mandra-1 clone, 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA), Alkaline Phosphatase (ALP) (monoclonal, 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA), Pax7 (monoclonal, 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA). Secondary antibodies used were: Alexa-Fluor 488 anti-rabbit (Invitrogen, Carlsbad, CA) and DyLight 549 anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). To demonstrate specificity of the p65 polyclonal antibody, antigen competition was carried out by pre-incubating anti-p65 overnight with either p65 (Abcam, Cambridge, MA) or a control peptide at a concentration of 40 $\mu\text{g/ml}$. Staining for monoclonal antibodies was verified using sections null for primary antibody.

Confocal Microscopy: Confocal microscopy was performed using a Zeiss 510 META confocal laser scanning microscope and images were collected using the LSM image acquisition software. EC and control muscle sections were imaged at the same magnification and microscope settings for analysis of signal intensity using a 40x oil immersion objective. Control muscles were imaged first and gain scan speed and offset were optimized for each channel. EC samples were imaged at the same settings as control samples. A 4 μm stack with a Z interval of 0.5 μm was scanned through the center of each section.

Data Analyses: All statistical analyses were computed using a SAS statistical software package (V9.2; SAS Institute, Cary, NC), with expression profiling data computed using Partek Genomics Suite (V6.5; Partek Incorporated, St. Louis, MO).

Muscle Function. Muscle strength, CK and soreness were analyzed with a repeated measures analysis of variance (ANOVA) and, when appropriate, Tukey's post-hoc analysis was performed. CK data were not normally distributed and were log transformed prior to performing the ANOVA. Significance was set *a priori* at <0.05 .

Microarray. Probe set means from microarrays were generated from the PLIER algorithm (typically 6 iterations) in Expression Console (Affymetrix Inc., Santa Clara, CA) and imported into Partek Genomics Suite software for statistical analysis. PLIER (Probe Logarithmic Intensity Error) is a model-based signal estimator beneficial to multi-array estimations. Data were \log^2 transformed and then analyzed by analysis of covariance (ANCOVA) using age and body mass index as covariates. The data outputs were stringently filtered on the basis of p-value ($p < 0.007$). Gene function and network analysis was performed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). IPA functional analysis was used to identify the molecular and cellular functions of the dysregulated gene set. Genes that met $p < 0.007$ and were associated with molecular and cellular functions in the Ingenuity Pathways Knowledge Base were considered for further analysis. Fischer's exact test was used to calculate a p-value determining the probability that each molecular and cellular function assigned to that data set was due to chance alone. Genes were classified in each set by function. The

genes involved in the functions sets of interest were further screened. Differentially expressed genes were then mapped to their corresponding genes in the Ingenuity Pathways Knowledge Base. These genes were then overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity. The IPA score is a negative log of the p-value for each network.

Immunofluorescent Analyses. Quantification of all immunofluorescent images was made using three randomly acquired fields from 15 subjects under both the control and EC conditions. Images were taken with a 40X objective to ensure accurate identification of nuclei origin (myonuclei or non-myonuclei) and co-localization status. At this magnification, approximately 8-10 myofibers per field were analyzed, corresponding to roughly 24-30 myofibers and 180 total nuclei per subject per condition (control and EC).

For our initial confocal-based quantification of p65 nuclear localization, triple immunostaining, with antibodies against dystrophin, p65 and Topro3 for nuclei were used. The 3 channels (dystrophin, p65, Topro3) from each stack were merged to determine the myofiber boundary. Images were analyzed using Zeiss LSM software. Nuclei with a mass center inside the dystrophin ring were considered myonuclei, and nuclei with their mass center outside of the dystrophin ring were considered non-myonuclei. The number of p65⁺/Topro3⁺ myonuclei and non-myonuclei were counted and expressed as a percentage of total myonuclei and non-myonuclei, respectively in both control and EC samples for each subject. For each stack, the determination of p65⁺/Topro3⁺ nuclei was made based

on the persistence of co-localization through the entire stack (8 slices). To further assess the specific cellular compartment of NF- κ B activation, we performed IHC using two commonly applied pericyte markers: NG2 proteoglycan (NG2) and alkaline phosphatase (ALP), and Pax7 for satellite cells. Images were analyzed using Metamorph software (Molecular Devices, Downington, PA). P65⁺ nuclei (p65⁺/DAPI⁺) that stained positive for NG2, ALP or Pax7 were counted and expressed as a percentage of total p65⁺ nuclei in each condition for each subject. To be considered as a Pax7⁺ cell, Pax7 had to co-localize to the nucleus. immunofluorescent enumerations were carried out by the same investigator, who was blinded to the condition (EC or control). Data were analyzed using a paired Student's t-test with a SAS statistical software package. Significance was set *a priori* at $p < 0.05$.

Results

Muscle biopsy samples from 35 subjects were analyzed by microarray to examine differentially expressed genes 3 h following EC. Of the 35 subjects, 30 had sufficient mRNA yield to perform subsequent qRT-PCR confirmational analysis and 15 of the 35 subjects had sufficient tissue for immunohistochemical (IHC) and nuclear protein extract (transcription factor ELISA) analyses. Data for the indirect markers of muscle damage are presented for both the whole- (n=35) and sub-cohorts (n=15) to demonstrate that the sub-cohort of 15 subjects had similar levels of muscle injury and were representative of the larger whole cohort.

Indirect markers of muscle damage: Decreased muscle strength, elevated creatine kinase (CK) activity and elevated muscle soreness following EC indirectly indicated a moderate level of muscle damage in both subject cohorts. Overall, mean muscle strength in both the whole- and sub-cohorts was significantly reduced from baseline levels following EC as indicated by a significant main effect of time ($p < 0.01$) (Figure 3.1A). Post-hoc analysis showed lower strength levels compared to the baseline strength level at all time points post-EC except for 120 h post-EC. There were no differences between the cohorts. Mean serum CK activity levels were significantly ($p < 0.01$) elevated from baseline after EC (Figure 3.1B) and demonstrated large variability (62-20,078 U·L⁻¹ at 120 h post-EC), driven primarily by two high responding subjects. Post hoc analysis showed that CK activity was significantly elevated at 24, 48 and 72 h following EC compared to baseline in both cohorts. There were no significant differences between the cohorts for CK activity. Muscle soreness was significantly elevated from baseline after EC in both cohorts as indicated by a significant main effect of time ($p < 0.01$) (Figure 3.1C). Post-hoc analysis showed elevated soreness levels in both cohorts compared to the baseline level at all time points except for 120 h post-EC. There were no significant differences between the two cohorts at any time point.

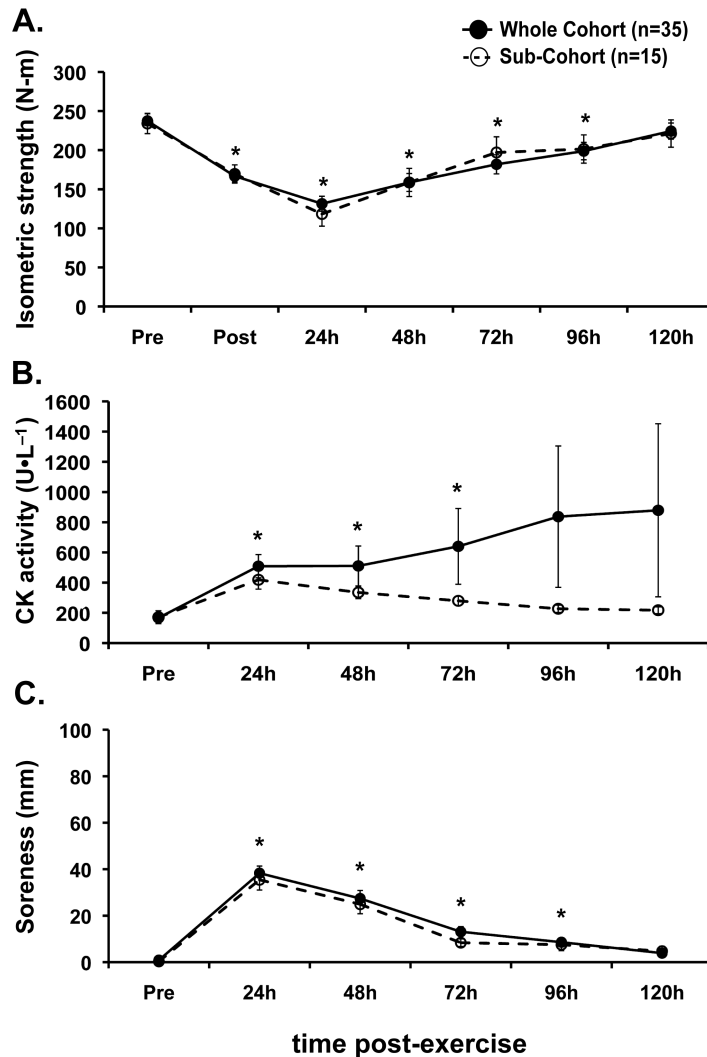


Figure 3.1: Eccentric contractions (EC) result in muscle damage. A) Mean isometric strength pre-, post- and for 120 h following EC in the whole cohort (n=35) and the sub-cohort (n=15), whose tissue was used for subsequent IHC and ELISA analyses. B) Mean serum CK activity pre-ECC and every 24 h for 120 h following EC in both cohorts. C) Mean muscle soreness pre-ECC and every 24 h for 120 h following EC in both cohorts. Data are mean \pm SE. * indicates significant difference from pre-ECC levels for both cohorts ($p < 0.05$).

Global gene expression and molecular network analysis implicate NF- κ b signaling

following EC: To assess transcriptional changes in response to EC, we compared expression profiles in the muscle biopsies obtained from the EC leg vs. control (non-EC) leg. For our network level analysis, we chose to use a p value cutoff of $p < 0.007$ as our

inclusion criteria, rather than fold change, to adequately represent small, potentially important transcriptional changes. Significantly dysregulated genes were analyzed by IPA software (Ingenuity Systems) to assess functional pathways and associated genes that are modified as a result of eccentric contractions. IPA analysis revealed multiple high scoring gene networks with relevant functions relating to inflammatory and immunologic response, cell growth and proliferation and cell signaling. We noted that the transcription factor nuclear factor-kappaB (NF-kB) was highlighted in several of these networks and therefore asked IPA to build a network based on known associations between NF-kB and the differentially regulated genes in our data set. Presented in Table 3.2 is a list of transcripts that were altered as a result of EC and identified by IPA as having a known association with the NF-kB transcription factor complex. Collectively, the network level analyses suggested that activation of NF-kB may be an important molecular response to EC.

qRT-PCR analysis confirms select genes: To verify microarray data, we chose to confirm, via qRT-PCR, 3 of the more modestly altered transcripts that showed up in our NF-kB networks. These transcripts included: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (*NFKBIA*, also known as *IkBα*), inter-cellular adhesion molecule 1 (*ICAM1*) and CCAAT/enhancer-binding protein, delta (*CEBPD*). We performed qRT-PCR on samples from the EC and control legs of 30 subjects and expression was normalized to our housekeeping gene *B2M*. Figure 3.2 shows that gene expression changes, as measured by qRT-PCR were similar to the changes measured by the microarray, demonstrating its general reliability in our well-powered sample.

Table 3.2: NF- κ B-related transcripts that were altered as a result of eccentric contractions

Gene symbol	Gene name	Fold change	P	Relationship to NF- κ B
Growth factors/cytokines/chemokines				
<i>CCL2</i>	Chemokine (C-C) ligand 2	17.9	<0.001	NF- κ B involved in expression of <i>CCL2</i> mRNA
<i>IL18</i>	Interleukin 18	9.8	<0.001	IL18 increases translocation of NF- κ B to nucleus
<i>LIF</i>	Leukemia inhibitory factor	2.1	0.001	NF- κ B involved in expression of <i>LIF</i> mRNA
<i>CXCL1</i>	Chemokine (C-X-C) ligand 1	6.0	<0.001	CXCL1 increases activation of NF- κ B complexes
<i>CSCL2</i>	Chemokine (C-X-C) ligand 2	2.7	<0.001	NF- κ B involved in expression of <i>CXCL2</i> mRNA
<i>IL6</i>	Interleukin 6	1.8	<0.001	activation of NF- κ B increases expression of IL6
<i>VEGFA</i>	Vascular endothelial growth factor A	1.6	<0.001	activation of NF- κ B increases expression of VEGFA
<i>TGFB2</i>	Transforming growth factor, β 2	7.8	<0.001	TGFB2 involved in activation of NF- κ B complexes
<i>IFNK</i>	Interferon, κ	-2.2	<0.001	NF- κ B increases induction of IFNK
Transmembrane receptors				
<i>IL1R1</i>	Interleukin 1 receptor, type 1	7.3	<0.001	IL1R1 mediates NF- κ B DNA binding activity
<i>IL1RL1</i>	Interleukin 1 receptor-like, type 1	11.4	<0.001	IL1RL1 increases translocation of NF- κ B to nucleus
<i>IL1RL2</i>	Interleukin 1 receptor-like, type 2	1.7	<0.001	IL1RL2 involved in activation of NF- κ B complexes
<i>RELT</i>	RELT tumor necrosis factor receptor	6.0	<0.001	RELT increases activation of NF- κ B complexes
<i>ICAM1</i>	Intracellular adhesion molecule 1	1.9	<0.001	NF- κ B activation increases expression of ICAM1
<i>VCAM1</i>	Vascular cell adhesion molecule 1	2.5	<0.001	NF- κ B activation increases expression of VCAM1
<i>TNFRSF12A</i>	Tumor necrosis factor receptor superfamily 12A	9.9	<0.001	TNFRSF12A increases activation of NF- κ B
<i>TNFRSF10B</i>	Tumor necrosis factor receptor superfamily 10B	1.7	<0.001	TNFRSF10B mediates activation of NF- κ B
Transcription factors				
<i>IFI16</i>	Interferon, γ -inducible protein 16	1.9	<0.001	IFI16 increases activation of NF- κ B complexes
<i>CEBPD</i>	CCAAT/enhancer binding protein, δ	2.4	<0.001	CEBPD expression dependent on NF- κ B activity
<i>ATF3</i>	Activating transcription factor 3	56.4	<0.001	NF- κ B increases induction of ATF3 protein
<i>NFKBIZ</i>	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ζ	2.9	<0.001	NFKBIZ protein increases activity of NF- κ B
<i>MYC</i>	v-myc myelocytomatosis viral oncogene homolog	25.2	<0.001	NF- κ B in nuclei increases MYC protein in nuclei
<i>HIF1A</i>	Hypoxia inducible factor 1, α subunit	1.7	<0.001	NF- κ B involved in expression of HIF1A protein
<i>JUN</i>	jun proto-oncogene	2.6	<0.001	Binding of NF- κ B complexes and JUN occurs
Other				
<i>NFKB1A</i>	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α	-1.3	0.006	NFKB1A decreases expression of NF- κ B complexes
<i>SOD2</i>	Superoxide dismutase 2	2.1	<0.001	NF κ B increases expression of SOD2 protein
<i>TRAFD1</i>	TRAF-type zinc finger domain containing 1	4.6	<0.001	TRAFD1 protein decreases activation of NF κ B
<i>CAMK2D</i>	Calcium/calmodulin-dependent protein kinase 2, δ	1.5	<0.001	CAMK2D protein increases the activation of NF κ B

(continued on next page)

Table 3.2 (continued)

Gene symbol	Gene name	Fold change	P	Relationship to NF- κ B
<i>SOCS3</i>	Suppressor of cytokine signaling 3	8.6	<0.001	NF κ B is involved in expression of SOCS3 protein
<i>MAP2K3</i>	Mitogen-activated protein kinase kinase 3	2.2	<0.001	MAP2K3 protein increases NF κ B activity
<i>HMOX1</i>	Heme-oxygenase 1	1.5	<0.001	NF- κ B involved in expression of HMOX1 protein

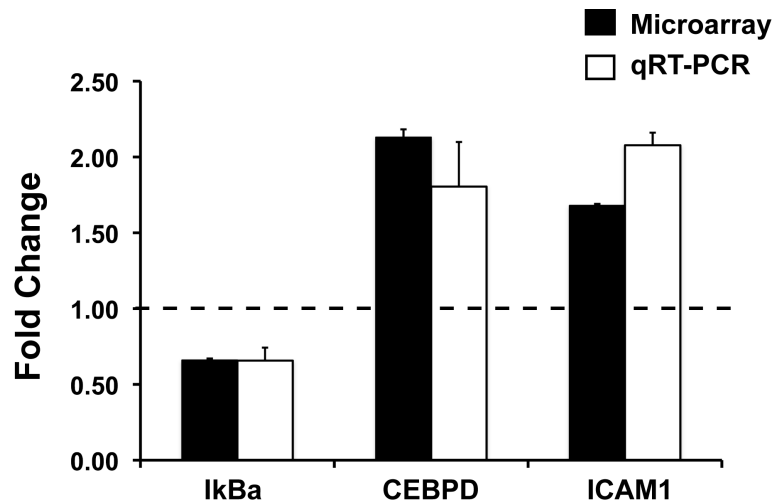


Figure 3.2: qRT-PCR confirmation of a subset of differentially regulated genes from the microarray experiment. Data are presented as mean fold change compared to the control (non-eccentrically exercised) leg. All PCR was significantly different at $p < 0.01$. Dotted line indicates control level (no change). Data are mean \pm SE. $n=35$ for microarray, $n=30$ for qRT-PCR.

Activation of NF- κ B following EC: To follow up on the observations made by our network analysis, we further hypothesized that NF- κ B was activated following EC. By transcription factor ELISA analysis, we found that there was a significant ($p=0.018$) 1.6 fold increase in NF- κ B DNA binding activity in muscle samples from the EC vs. the non-EC control leg (Figure 3.3A). To further confirm this finding and to gather additional information on the location of NF- κ B activity, we performed immunofluorescence and

confocal microscopy on serial muscle sections of EC and control samples using antibodies against the p65 subunit of NF- κ B, dystrophin and Topro3. This allowed us to measure the localization of p65 to individual nuclei (p65⁺/Topro3⁺) as an indirect measure of NF- κ B activation. To determine whether NF- κ B activity was localized to nuclei within myofibers (myonuclei) or in the interstitial spaces, we co-stained sections with an antibody against dystrophin to distinguish the myofiber boundary. Confocal imaging of our stained sections revealed a p65 signal with intense foci localized outside of the myofiber boundary as well as a more diffuse pattern of expression within the myofiber (Figure 3.3C). The absence of the p65 signal, particularly at defined interstitial foci, in the presence of the peptide used for immunization, demonstrates specificity of the antibody (Figure 3.3B). Quantification of p65⁺ nuclei showed a significantly ($p=0.039$) greater percentage of non-myonuclei that were p65⁺ in the EC leg (24%) compared to the control leg (13%) (Figure 3C and D). Overall, increases in p65⁺ nuclei in the EC vs. control leg were consistent with the DNA binding results. Interestingly, we found that, in general, expression of p65 was very low within individual myofibers (inside the dystrophin ring) and that localization of p65 to myonuclei was a rare occurrence.

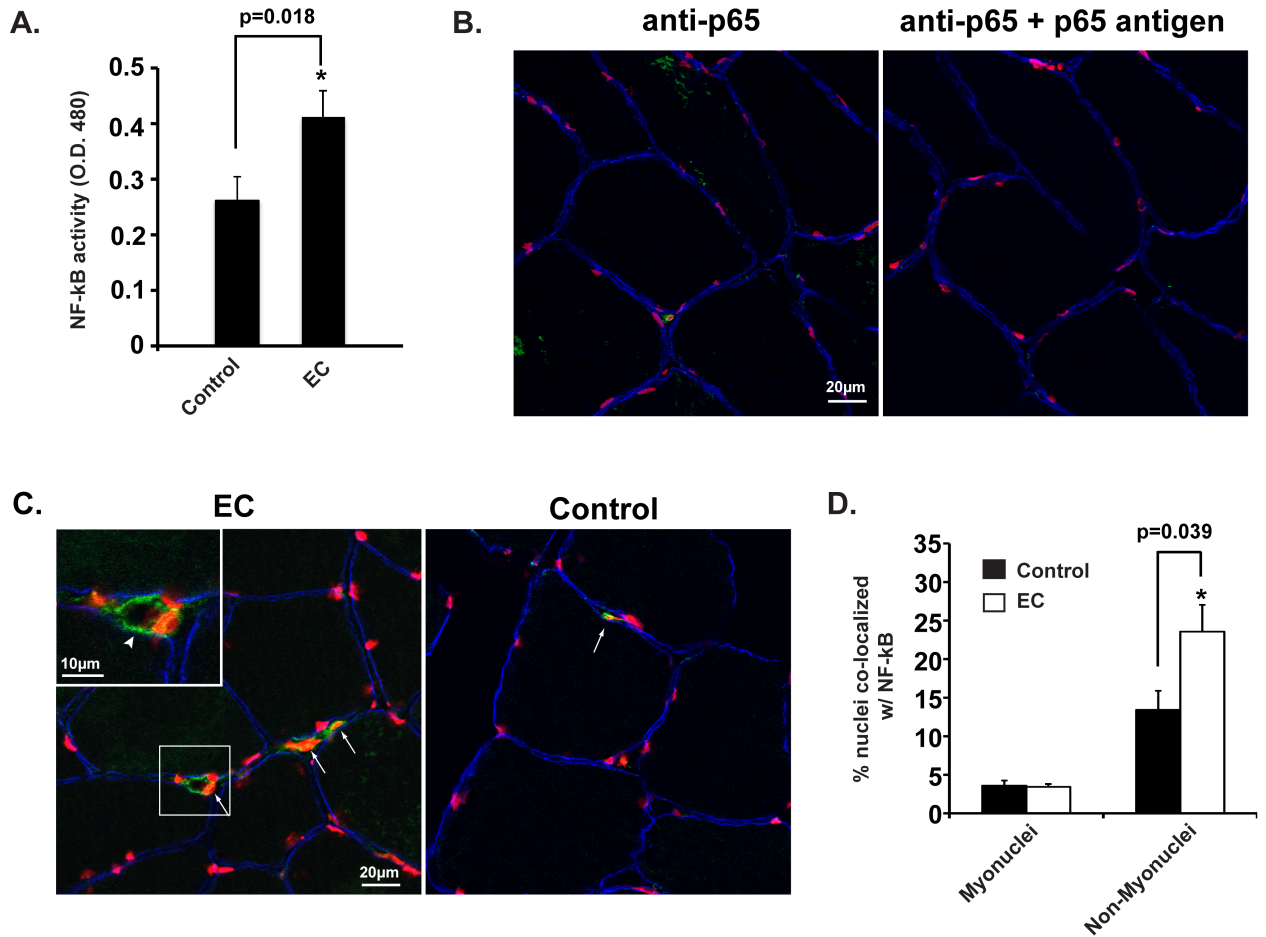


Figure 3.3: Activation of NF- κ B 3 h following eccentric contractions (EC). A) NF- κ B (p65) activity measured in EC and control nuclear extracts by an ELISA-based DNA binding assay. Data are presented in arbitrary absorbance units (450nm). n=15. Data are means \pm SEM. B) Specificity of the p65 antibody is confirmed by an antigen competition experiment. Confocal images of 10 μ m serial sections of an EC muscle sample using antibodies against p65 (green) in the presence of a control peptide (left panel) or the p65-derived peptide (right panel), dystrophin (blue), and nuclei (topro=red). C) Confocal images of triple stained 10 μ m sections from representative EC and control samples for dystrophin (blue), the p-65 subunit of NF- κ B (green) and nuclei (topro=red). Arrows indicate nuclei that were determined to be p65 positive (p65+/topro3+). White square in the EC image denotes the boundaries of the inset image. Arrowhead in the inset image indicates potential location of microvessel. D) p65+/topro3+ myonuclei and non-myonuclei expressed as a percentage of total myonuclei and non-myonuclei. n=15. * indicates significant difference (p<0.05) between control and EC muscle samples.

NF-kB activation in NG2 and ALP positive cells, but not Pax7 positive cells: Our initial confocal analysis indicated that the cellular origin of NF- κ B activity (p65+/Topro3+) was

localized to the interstitial spaces between fibers. Furthermore, it was noted that a high number of these p65⁺/Topro3⁺ nuclei were positioned adjacent to, or nearby, what we perceived to be potential sites of microvasculature, as indicated by a tubular p65 staining pattern (arrow in Figure 3.3C inset image) between fibers. This suspicion led us to hypothesize that NF-kB was localized to the nuclei of cells adjacent to, or associated with the microvasculature. Thus, we co-stained serial muscle sections with markers of vessel-associated pericyte cells (NG2, ALP) and satellite cells (Pax7), as the latter have been commonly observed in close proximity to capillaries (19). NG2 is a reliable pericyte marker that has been used frequently to discriminate pericytes in a variety of different tissues and has also been shown to effectively differentiate between pericyte and endothelial cells (20). Similarly, ALP has been used previously as a pericyte marker, specifically in muscle tissue (21). Co-staining with these markers revealed that both NG2 and ALP co-localized to 69% and 64% of p65⁺/DAPI⁺ nuclei, respectively (Figure 3.4A, B and D). In contrast, Pax7 co-localized to only 1% of p65⁺/DAPI⁺ nuclei (Figure 3.4C and D). These data suggest that pericyte cells and not satellite cells are a primary source of increased NF-kB activity in muscle following eccentric contractions.

Because eccentric contractions induce a degree of skeletal muscle damage and result in a well-studied muscle progenitor cell response (10, 22, 23), we were further interested in determining whether there were changes in the relative number of satellite cells 3 h following EC. We found that approximately 5% of total nuclei stained positive for Pax7 and that there were no differences between EC and control muscle for total satellite cell number (Figure 3.4F). This finding is consistent with previous reports, both in terms of

the relative number, and the absence of significant numerical changes at approximately 3 h post-EC in human muscle (10, 12). We were also interested in performing a preliminary quantification of pericyte number in human skeletal muscle under the control and EC conditions, as there are currently no data on pericyte number in human skeletal muscle. By averaging the mean number of NG2⁺ and ALP⁺ cells in both conditions, we found no changes in the relative number of pericyte cells following EC (Figure 3.4E). Interestingly, our enumerations indicated that roughly 7% of nuclei (in both conditions) stained positive for pericyte markers. Further analyses, for example, co-staining with multiple pericyte markers and subsequent quantification of double labeled cells will be important in more definitively ascertaining pericyte cell population in humans skeletal muscle.

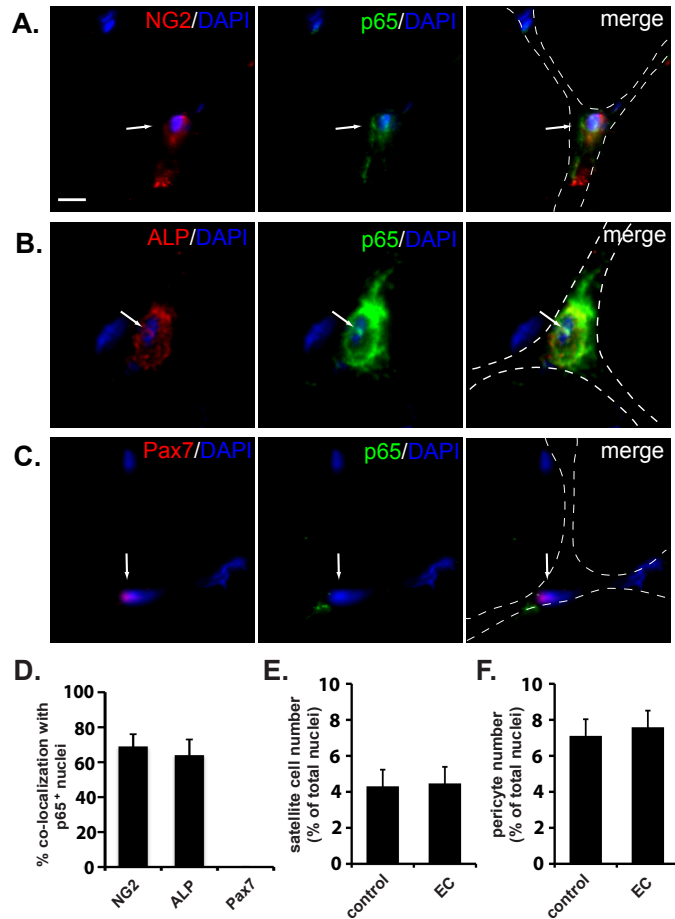


Figure 3.4: Localization of NF-kB to pericytes and not satellite cells 3 h following eccentric contractions (EC). A) Immunofluorescent images of a triple stained 10µm section from an EC sample for DAPI (blue), the p65 subunit of NF-kB (green) and NG2 (red). Arrow indicates nucleus that has been determined to co-localize with NF-kB (p65⁺/DAPI⁺) and stain positive for NG2. B) Immunofluorescent images of a triple stained 10µm section from an EC sample for DAPI (blue), the p65 subunit of NF-kB (green) and alkaline phosphatase (ALP) (red). Arrow indicates nucleus that has been determined to co-localize with NF-kB (p65⁺/DAPI⁺) and stain positive for ALP. C) Immunofluorescent images of a triple stained 10µm section from an EC sample for DAPI (blue), the p65 subunit of NF-kB (green) and Pax7 (red). Arrow indicates a Pax7⁺ cell that does not co-localize with p65. D) Relative number of NG2, ALP and Pax7 positive cells that were p65⁺/DAPI⁺, reported as a percentage of total myonuclei per field. E) Relative number of cells determined to be satellite cells, based on Pax7 staining in control and EC muscle sections. F) Relative number of cells determined to be pericytes, based on NG2 and ALP staining in control and EC muscle sections. Dotted lines in merged images denote the approximate myofiber boundary based on phase contrast images. Scale bar = 10µm

Discussion

The primary objective of this study was to uncover potentially important gene groups and/or molecular networks that govern the early response (3 h post) to EC in humans. We positioned ourselves to take a systems approach by using whole genome analysis in a well-powered sample of human subjects following EC. Our network analysis led us to identify the involvement of NF-kB activation in the early response of muscle to EC. Moreover, to further understand the potential impact that NF-kB signaling may have in this early response, we have also localized its activity primarily to muscle-residing pericyte cells.

Our finding of increased NF-kB activation following EC was supported by evidence from both DNA binding and immunohistochemical experiments. We became interested in NF-kB activation when our network analysis identified several significant associations between many of our differentially expressed genes and activation of the NF-kB transcription factor complex. Most notably, we observed significant, and in some cases large, alterations in mRNA expression of cytokines, growth factors, transcription factors and membrane receptors that have been shown to function both upstream and downstream of NF-kB activation. To our knowledge, we are the first to demonstrate an increase in NF-kB activity following EC. In fact, there are very few reports in humans on the effects of exercise or muscle contraction (concentric or eccentric) on NF-kB activation in muscle tissue. This is somewhat surprising in light of the fact that EC results

in a predictable and well-characterized inflammatory and oxidative stress response (24, 25), both of which are associated with activation of NF- κ B (26). The few studies that have measured NF- κ B activation in human muscle following an exercise have done so subsequent to acute bouts of resistance or endurance type exercise and have reported either an increase (27) or no change in its activity (28). However, it must be noted that the two referenced studies differed from the current study in the mode of exercise and the timing of tissue extraction, making a direct comparison difficult. Perhaps most relevant to our data, Durham et al. (28) reported decreased NF- κ B DNA binding activity via electrophoretic mobility shift assay immediately following a lower body resistance exercise (eccentric and concentric work). Interestingly, they also provide data 1 h post-exercise that showed a return of NF- κ B DNA binding activity back to basal levels. Given our results of increased NF- κ B activity 3 h post-EC, we speculate that perhaps NF- κ B activation is decreased in the initial moments post-exercise and increased in the subsequent hours of recovery. In contrast to the previous human work, but consistent with our data, studies in rodents have demonstrated a clear pattern of NF- κ B activation following muscle contraction and/or an acute bout of exercise (29-31). For instance, downhill treadmill running in rats, which has a strong eccentric component, has been shown to increase NF- κ B DNA binding activity and enhance the expression of genes under NF- κ B regulation (29, 31).

To more deeply probe the functional significance of NF- κ B activity in skeletal muscle following EC, we believed it was important to localize the origin of its activity, as its effect may be a function of its cellular compartmentalization. For example, NF- κ B has

traditionally been considered to primarily have anti-myogenic effects in regenerating and developing muscle (17, 32). However, Dahlman *et al.* (33) have recently provided compelling evidence that NF- κ B activation in muscle-residing fibroblasts can promote myoblast proliferation and enhance myogenesis via a nitric oxide synthase (iNOS) mechanism in developing mouse muscle. Thus, NF- κ B dependent transcription may have distinct roles in myogenesis, depending on the origin of its activity. This finding may be particularly relevant to our EC model, given that 1) muscle development largely recapitulates the molecular events of muscle regeneration following damage (1) and 2) recovery from EC involves the activation of satellite cells and a significant regenerative response (11, 34). Our initial confocal analysis indicated that the majority of NF- κ B expression and nuclear localization originated not from the myofibers but from the interstitial spaces, specifically from cells adjacent to, or nearby what we suspected could be microvessel structures. Therefore, we explored the possibility that NF- κ B was activated in cells directly associated with (pericytes), or positioned in close proximity to (satellite cells), the microvasculature (19). This led to the finding that the majority of NF- κ B activity derived from cells that were positive for pericyte markers. Pericytes are stem cell-like constituents of blood microvessels and regulate functions of vascular development and remodeling (35). They can be readily distinguished in electron micrographs of muscle samples (36); however, the functional roles that they may play in muscle, particularly in the periods following eccentric contractions is unknown.

A well-supported potential function for pericyte specific NF- κ B activation following EC, particularly considering its early activation (3 h), is in the signaling of an inflammatory

response. It has been established that EC results in a predictable inflammatory response (25) and that this response is most likely necessary for effective regeneration of damaged tissue (25, 37, 38). Muscle resident pericytes are strategically positioned along the vessel walls to efficiently signal a systemic response. While there are no data on the signaling properties of pericyte cells following EC, there is evidence to support a role for endothelial cells in the signaling of inflammatory cells via chemokine (c-c motif) ligand 2 (CCL2, also known as MCP1)(39), which is regulated by NF-kB activity (40). In support of this role, we have reported a marked 17.9 fold increase in *CCL2* expression via microarray. Additional support for a potential inflammatory signaling role is evident in our microarray data set, which highlights alterations in several transcripts associated with inflammation, for example, the cell adhesion molecules inter-cellular adhesion molecule 1 (*ICAM-1*) and vascular cell adhesion protein 1 (*VCAM*), as well as several interleukins and interleukin receptors (IL6, IL18, IL1R1, IL1RL1 and IL1RL2). We have also reported increases in expression of CCAAT/enhancer-binding protein delta (*CEBPD*) via qRT-PCR. This finding, a result that we have observed in two previous human EC studies (13, 41), lends further support to an NF-kB mediated inflammatory response following EC in humans. CEBPD, an inflammatory-related transcription factor, is indirectly regulated by NF-kB (42) and moreover, has been shown to regulate the expression of CCL2 in vascular smooth muscle (43).

Several recent reports have also provided compelling evidence to support a role for both muscle and non-muscle derived pericytes in myogenesis and the restoration of dystrophin in *mdx* mice (21, 44, 45). The myogenic potential of pericytes, along with the activation

of a major signaling pathway (NF- κ B) associated with muscle regeneration (33), may suggest a possible role for pericytes in the regenerative response that follows eccentric contractions in humans (10, 11).

Due in large part to this possibility, we were interested in whether we could detect a potential proliferative response by measuring the relative number of pericytes in both EC and control muscle samples. We were unable to find such a difference. Though our data shed very little light on a potential function for pericytes in the regenerative response following EC, we were intrigued to find that approximately 7% of total nuclei per field (approximately 8-10 myofibers) stained positive for markers of pericytes. To our knowledge, we are the first to report data on the relative pericyte abundance in human skeletal muscle cross-sections. However, we must acknowledge that quantification of pericyte number in human muscle was a secondary aim, facilitated by our NG2 and ALP labeled cross sections. Follow-up work will be important to confirm this finding.

Consistent with a previous report (12), we were also unable to find a significant difference in satellite cell number at 3 h post EC. We interpret this finding, not as evidence to support the lack of a regenerative response as a consequence of our EC protocol, but rather, as insufficient time to allow for measurable changes to occur. This assertion is supported in a recent study by McKay *et al.* (12) who used a similar knee extensor protocol and showed a significant satellite cell response using the Pax7 marker at later time points (24, 72 and 120 h), but not at 4 h post.

In summary, our well-powered systems analysis of gene expression changes following an eccentric contraction protocol in humans allowed us to make some revealing findings.

We have reported the first evidence of NF-kB activation in human muscle following eccentric contractions. Furthermore, we were able to localize the majority of that activity to muscle resident pericytes and have thus implicated a novel and potentially important role for these cells in the response to eccentric contractions in humans. We have also generated a significant set of data comprising original and previously reported differentially expressed genes from which to develop additional hypotheses. Future work will be important in deciphering the functional significance of pericyte-specific NF-kB activity in muscle damage, regeneration, and adaptation.

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CHAPTER IV

NF-KB ACTIVITY FUNCTIONS IN PRIMARY PERICYTE CELLS IN A CELL- AND NON-CELL-AUTONOMOUS MANNER TO AFFECT MYOGENESIS

Introduction

Skeletal muscle is remarkably proficient at regenerating in response to changing functional demands and/or damage. Given its essential roles in locomotion, heat production and energy storage, the regenerative capacity of skeletal muscle is essential to normal healthy function. The complex mechanisms that regulate the regenerative response rely on intricate interactions and signaling patterns between the regenerating myofibers, muscle progenitor cells (MPCs), the interstitial environment, and other systemic factors.

Skeletal muscle is a heterogeneous mix of multi- and mono-nucleate cell types. In addition to the multi-nucleate myofibers, several mono-nucleate cells have been identified that normally reside in muscle or infiltrate the tissue following injury. While we still know relatively little about the functional interactions between these distinct cell types, it is clear that they play an integral role in the acute response and subsequent regenerative process following damage. For example, evidence is accumulating to support the hypothesis that inflammatory cell signaling plays a central role in muscle regeneration following injury (26). Likewise, signaling relationships have been shown to occur between muscle residing fibroblasts and myoblasts to enhance myogenesis during muscle development and following muscle injury. Mathew *et al.* (21) have shown that the

fibroblast specific transcription factor tcf4 regulates paracrine signaling from fibroblasts to myoblasts to enhance muscle fiber formation. Similarly, Dahlman *et al.* (9) have recently reported that stromal fibroblasts regulate fusion of surrounding myoblasts via nitric-oxide synthase expression under the control of the transcription factor nuclear factor-kappaB (NF-kB). Thus, it is becoming increasingly apparent that paracrine signaling from the cellular milieu of skeletal muscle is important in the regulation of muscle regeneration.

Recently we have identified NF-kB activation in skeletal muscle resident pericyte cells as a potentially important molecular event following eccentric contractions in humans, a stimulus known to produce transient muscle injury and subsequent regeneration (18; Chapter 3). Therefore, the current study was undertaken to explore the functional significance of this finding in the context of muscle regeneration, using an *in vitro* paradigm of myogenesis. I first sought to determine if altered NF-kB activity in cultured primary pericytes affected proliferation and/or differentiation of co-cultured skeletal muscle myoblasts. Consistent with the emerging anti-myogenic role of NF-kB (2), we hypothesized that NF-kB activation in primary pericytes would enhance local myoblast proliferation and inhibit differentiation. Furthermore, in light of recent evidence to support the myogenic potential of pericyte cells (8, 11, 23), we were also interested in determining how altered NF-kB activity affected this potential in cultured primary pericytes. We hypothesized that NF-kB activation in cultured primary pericytes would decrease their myogenic potential.

In the following study we provide additional supporting evidence for the myogenic capacity of pericyte cells and a novel role for NF- κ B activation in this response. We also provide evidence of a potential NF- κ B dependent signaling relationship between primary pericytes and skeletal muscle myoblasts to regulate proliferation and myotube formation.

Methodology

Cell Culture: Human Primary Pericytes (HPP) were obtained from PromoCell (Heidelberg, Germany), where they were isolated from placental tissue and reported as positive for the mesenchymal stem cell markers CD146 and CD105, and negative for endothelial markers CD31 and CD34. HPPs exhibited a distinct stellate morphology with many long cytoplasmic processes, consistent with previously published pericyte descriptions. Doubling time *in vitro*, calculated by linear regression (cell number vs. time), was determined to be approximately 40 h under the conditions used in this study. Cells were passaged up to 10 times, during which there was no observed trend towards senescence at these passage numbers. Cultures were maintained at 37°C in a 5% CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (GM) and 1% penicillin and streptomycin. Before cultures reached 90% confluence they were passaged using 0.25% trypsin, 0.1% EDTA. Cells used for all experiments were between passages 3-8. C₂C₁₂ myoblasts were obtained from the American Type Culture Collection (ATCC) and were cultured under the same conditions as HPPs.

Transient Transfections: Plasmid DNA was transfected into HPPs using a Basic Nucleofector Kit for Primary Smooth Muscles (Amaxa, Gaithersburg, MD). We used the following expression plasmids: dominant negative (d.n.) IKK β (K44M), constitutively active (c.a.) IKK β (S177/S188 \rightarrow EE) and an empty vector of the expression plasmid (pEF6/HisB; Invitrogen, Carlsbad, CA). The d.n. IKK β plasmid was developed in the lab of Michael Karin (University of California, San Diego, CA) and encodes a kinase-dead form of IKK β . The c.a. IKK β was developed in the lab of Steve Shoelson (Joselin Diabetes Center, Boston, MA) and encodes a constitutively active form of IKK β . To visualize expression of the transfected proteins, the d.n. IKK β and c.a. IKK β vectors were subcloned into the N-terminus of an EGFP expression vector (pEGFP-n1; Clontech, Palo Alto, CA) by the Kandarian lab (Boston University, Boston, MA) to create the d.n. IKK β -EGFP and c.a. IKK β -EGFP fusion proteins. 10 μ g of each experimental plasmid plus 5 μ g of reporter plasmid (Clontech, Mountain View, CA) were electroporated into 5X10⁵ HPPs with a Nucleofector II machine (Amaxa, Gaithersburg, MD) using the P-13 program. Following electroporation, HPPs were either seeded onto 60-mm plates or slide flasks either alone or in co-culture with C₂C₁₂ myoblasts or in monoculture.

Luciferase Reporter Assay: HPPs were co-transfected with 5 μ g of the pNF-kB-MetLuc2-Reporter plasmid (Clontech, Mountain View, CA) in conjunction with each of the IKK β plasmids as described previously. Twenty-four h post-transfection, medium was collected in each condition and luciferase activity was assayed using a Ready-To-Glow Secreted Luciferase Reporter Assay (Clontech, Mountain View, CA), according to the manufacturer's instructions. Luminescence measurements were carried out on a

FLUOstar Optima plate reader (BMG LABTECH, Offenburg, Germany). A pMetLuc2-Control Vector (Clontech), driven by constitutively active cytomegalovirus immediate early promoter, was used as a positive control for the constitutive expression of *metridia* luciferase. As a positive control for NF- κ B activity, we transfected HPPs with 5 μ g pNF- κ B-MetLuc2-Reporter plasmid and subsequently treated with 10ng TNF- α , a known stimulator of NF- κ B.

Primary Pericyte/Myoblast Co-Cultures: For all co-culture experiments, HPPs and C₂C₁₂ myoblasts were cultured at ratios between 1:5 and 1:8. In these situations, it was estimated that about 50% of transfected HPPs survived and adhered to the slide flask in these studies, leaving approximately 7.4x10⁴ cells per flask. C₂C₁₂ myoblasts were then seeded on to the slide flasks with the adherent HPPs at a density of 3.8x10⁵ cells in differentiation medium.

Myoblast and HPP Myogenic Differentiation: To assess myogenesis in the HPP/myoblast co-cultures, immunocytochemistry was used to determine myotube formation 96 h following the initiation of co-culture. Co-cultures were seeded as described above and incubated in differentiation medium (DMEM supplemented with 2% horse serum) for 4 d with medium changes every 2 d. Following 4 d of differentiation, cells were fixed for 2 min in 2% paraformaldehyde at RT. The cells were then permeabilized for 3 min with Karsenti's Lysis Buffer (0.5% Triton X-100, 80mM PIPES, 1.0 mM MgSO₄, 5.0 mM EGTA, pH 7.0) after which they were rinsed twice with phosphate buffered saline with 0.1% Tween-20 (PBST). Cells were then incubated with monoclonal MF20 antibody

against myosin heavy chain (Developmental Hybridoma Bank) for 60 min in the dark at room temperature. After 3 washes with PBST, the cells will be incubated with AlexaFluor 594 anti-mouse (Invitrogen, Carlsbad, CA) for 60 min in the dark. Cells were then washed again 3 times in PBST and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). Co-cultures were visualized and photographed using a Nikon model TMS inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI). Images were analyzed using Metamorph Imaging software (Molecular Devices, Downingtown, PA). Ten random images from each treatment (c.a. IKK β , d.n IKK β or empty vector control) were acquired and used for determination of both myotube nuclear number and fiber diameter. For myotube diameter measurements, approximately 100 diameters were randomly selected for analysis. Three lines running perpendicular to the orientation of the fixed myotubes were drawn at evenly spaced intervals across each field of the image using the Metamorph software. Diameter measurements were made in micrometers at all points where the line crossed a myosin heavy chain (MHC) positive myotube. All experiments were replicated 3 times.

Myoblast Proliferation in Co-Cultures: To assess proliferative capacity of myoblasts in co-culture with HPPs under the different conditions, cell number was determined 48 h and 96 h following the initiation of co-culture. Cells were visualized using DAPI and Metamorph Imaging software (Molecular Devices, Downingtown, PA) was used to count the number of cells in 10 randomly acquired images in each condition from 3 replicates.

HPP Myogenic Fusion: To determine if HPPs have the capacity to fuse to existing myotubes, HPP nuclei were labeled by incubating them in 5-Bromo-2'-deoxy-uridine (BrdU) for 48h. Labeled HPPs were then transfected with one of the 3 expression vectors (c.a. IKK β d.n IKK β or empty vector control) and cultured onto 4 d differentiated C₂C₁₂ myotubes. Co-cultures were incubated in differentiation medium for an additional 3 d. HPP nuclei were then detected using a BrdU Labeling and Detection Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Briefly, co-cultures were fixed with an ethanol fixative. Following fixation, slides were washed and incubated with Anti-BrdU solution for 30 min at 37°C. Slides were then washed again and incubated with AlexaFluor 594 anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) and 49,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) for 30 min at 37°C. Slides were once again washed, air dried and mounted. Stained slides were imaged on a Nikon model TE-2000 inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI). Determination of HPPs incorporated into myotubes was made based on the presence of a BrdU labeled nucleus within a differentiated myotube. Ten random images from each condition (c.a. IKK β , d.n IKK β or empty vector control) were acquired from 3 separate experiments. To avoid false positives due to the pericyte lying underneath or on top of the myotube, visual evidence of the myonucleus also had to be present in the phase contrast image.

Western Blots: Co-cultured cells were lysed in a RIPA buffer (20mM Tris pH7.5, 200mM NaCl, 0.25% Np-40, 1mM PMSF, 1mM Sodium Orthovanadate, 10mM NaF and analyzed for total protein concentration using the BCA Assay (Pierce, Rockford, IL). The

samples were then be boiled for 5 min. Equal amounts of protein from each sample were loaded into 4-15% gradient sodium dodecyl sulfate (SDS) poly-acrylamide gels (Bio-Rad Laboratories) and separated by electrophoresis (60-90min@100v). Fractionated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) at 100v for 1h. The membrane was then incubated in the appropriate primary antibody, at the appropriate dilution (5% BSA in 1XPBST) with rocking motion overnight at 4 degrees. Following the incubation in primary antibody, the membrane was incubated in the appropriate HRP-labeled secondary antibody (~1:1000) for 1.5 h. Membranes were treated with enhanced chemiluminescence (ECL) for two min, wrapped in plastic film, and exposed to Kodak film for the appropriate duration. Antibodies were: Rabbit polyclonal antibody for GAPDH obtained from Abcam (Cambridge, MA) and a mouse monoclonal antibody for MHC obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

Statistical Analyses: All analyses were carried out in 3 independent experiments. Means from each of these independent experiments were analyzed using a one-way ANOVA. Where there was a significant difference found, Tukey's Honest Significant Difference test was applied. Significance was set *a priori* at $p < 0.05$.

Results

Inducible expression of IKK β constructs: To alter NF- κ B activity in HPPs, we transfected them with either a constitutively active (c.a.) IKK β -EGFP, dominant negative (d.n.)

IKK β -EGFP, or an empty vector-EGFP (e.v.) expression plasmid. We estimated, based on EGFP fluorescence, that the transfection efficiency after 24 h was 50-60% (Figure 4.1A). To assess the effectiveness of the IKK β constructs to alter NF-kB activity in HPPs, we used a luciferase reporter assay. Twenty-four h post-transfection, luciferase activity was decreased by approximately 50% in the dominant negative IKK (d.n.) condition and increased approximately 2.5-fold in response to the constitutively active (c.a.) IKK (Figure 4.1B). As a positive control we also treated reporter-transfected cells with 10ng/ml tumor necrosis factor- α (TNF- α), a known stimulator of NF-kB activity. TNF- α treatment resulted in a 5-fold increase in NF-kB activity.

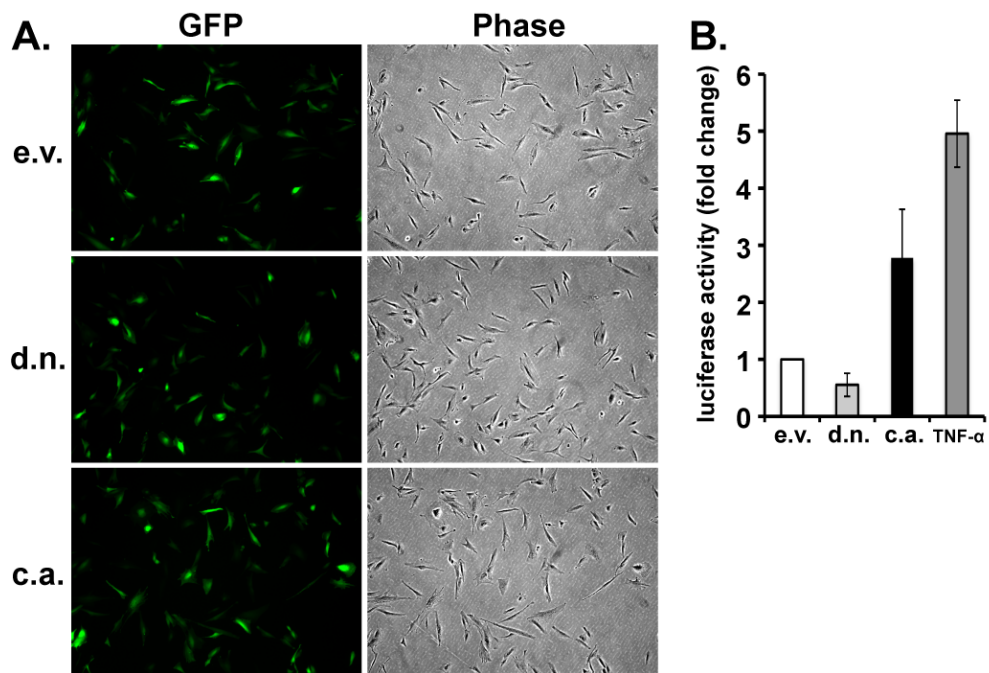


Figure 4.1: Altered activation of NF-kB in human primary pericytes (HPPs) following transient transfection of IKK β expression vectors. A) Representative images of HPPs 24 h following transfection with either an empty vector (e.v.), dominant negative IKK β -EGFP (d.n.), or constitutively active IKK β -EGFP (c.a.) plasmid. Left panel depicts GFP expression and the right panel is a phase contrast image. B) NF-kB activity as measured by a luciferase reporter 24 h following transfection with IKK β plasmids. An empty vector (e.v.) was used as a control. TNF- α , a known stimulator of NF-kB was used as a positive control. Data are means \pm SEM.

NF-kB activity alters myogenic fusion of HPPs to myotubes: In light of recent data (8, 22) that support the hypothesis that *in vitro* myogenesis of pericyte cells, including placenta-derived pericytes (23), we asked whether NF-kB activity is important in this response. Although HPP cultures varied in their morphology under different medium conditions, we were unable to verify that HPPs were myogenic in monoculture. This observation was based on the notable absence of myosin heavy chain (MHC) expression and the lack of apparent multi-nucleated cells following as many as 15 d in either a differentiation medium or conditioned medium from differentiated C₂C₁₂ myotubes (data not shown).

We next asked how NF-kB activity affected myogenic activity of HPPs in a recapitulation of the *in vivo* skeletal muscle environment, by culturing them on to differentiated myotubes. We labeled the nuclei of transfected HPPs with BrdU to distinguish them from other cells and cultured them onto 4 d old differentiated C₂C₁₂ myotubes. Following 3 d of co-culture we found evidence of HPP myogenic activity by the presence of BrdU-labeled HPP nuclei inside differentiated myotubes (Figure 4.2A), although overall, this was a very infrequent occurrence. In fact, by random image sampling (10 random images per condition from 3 separate experiments), we were only able to identify 7 instances (less than 1% of all HPP nuclei) of a BrdU-positive nucleus within a myotube. However, we did note that of these 7 occurrences, 5 of them were found in cells expressing the d.n. IKK β , suggesting that inhibition of NF-kB promotes myogenic fusion of HPPs. Because we found so few myogenic nuclei by random sampling, we scanned each slide entirely to identify the total number of myogenic HPPs among the three conditions. Among the three replicates, we found a significantly greater

number of myogenic HPPs in the d.n IKK β (23 ± 7.1) expressing cells compared to the e.v. (14 ± 4.4) and c.a. IKK β (10 ± 3.0) expressing cells. There were no differences between the e.v. and c.a. IKK β conditions for total number of BrdU-positive myonuclei. To get an indication of how frequently myoblasts fuse with differentiated myotubes under the same conditions, we co-cultured BrdU-labeled myoblasts at the same ratio onto 3 d differentiated myotubes. We found that there was approximately a 3-fold higher proportion of myoblasts fused to myotubes (Figure 4.2B).

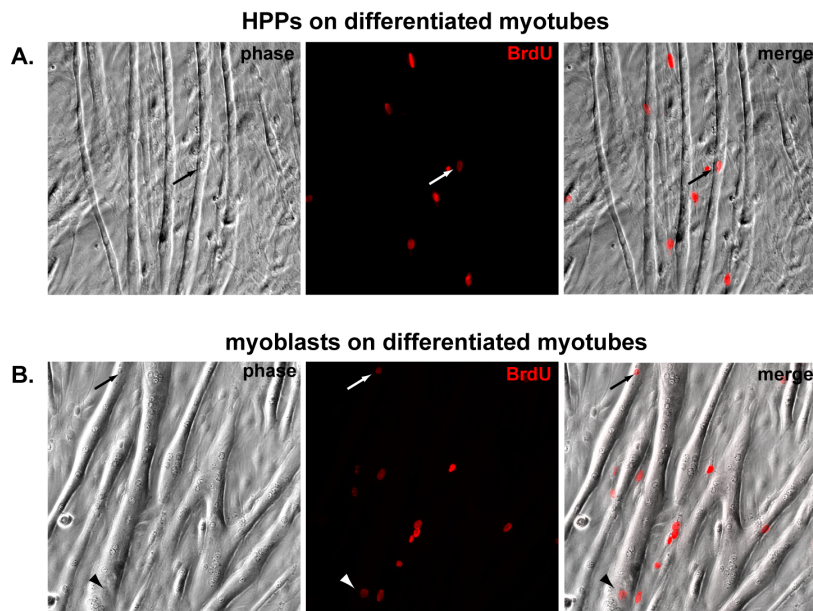


Figure 4.2: Human primary pericytes (HPPs) can fuse to differentiated myotubes. A) Images showing the fusion of an HPP, transfected with a dominant negative IKK β -EGFP (d.n.) expression plasmid to a differentiated myotube. HPPs were labeled with BrdU and co-cultured with differentiated myotubes for 3 d. Arrow indicates a BrdU-positive HPP nucleus that has fused with a differentiated myotube. B) Images showing the fusion of a BrdU-labeled myoblast with a differentiated myotube. The frequency of myoblast fusion was higher than that of HPPs following 3 d of co-culture. Arrow indicates a BrdU-positive myoblast nucleus that has fused with a differentiated myotube. Arrowhead indicates a BrdU positive myoblast that was determined to lie on top of or beneath a myotube because there is no evidence of the nucleus in the phase image. To be considered a myonucleus, evidence of the nucleus must have been clear in the phase image.

Pericyte specific NF-kB activity non-cell-autonomously regulates myoblast

differentiation: To determine if altered NF-kB activity in pericytes can extrinsically regulate myogenesis of local myoblasts, we co-cultured transfected HPPs with cycling C₂C₁₂ myoblasts that were then subjected to differentiation conditions. We observed a marked decrease in myotube development in the coculture c.a. IKK β expressing HPPs compared to cocultures with d.n. IKK β and e.v. IKK β expressing HPPs. This was confirmed by considerable decreases in MHC expression as measured by both immunofluorescence (Figure 4.3A) and Western Blot (Figure 4.3D). Overall, myotubes co-cultured with HPPs expressing the c.a. IKK β expression plasmid had fewer myonuclei (skewed distribution of myoneucli per myotube; Figure 4.3B) and a decreased myotube diameter (Figure 4.3C) than those that differentiated in the presence of HPPs expressing the e.v. or d.n. IKK β plasmids. Interestingly, we were unable to identify GFP-positive HPPs in the d.n. IKK β condition, suggesting that they had either died over the 96 h differentiation period or fused with myoblasts to form differentiated myotubes. However, because previous experiments had shown that d.n. IKK β expressing HPPs were viable beyond 96 h (data not shown), it suggested that they had fused to myotubes and the GFP signal became to diffuse within myotubes to detect. This would support our finding that suppression of NF-kB activity cell-autonomously enhances their myogenic activity.

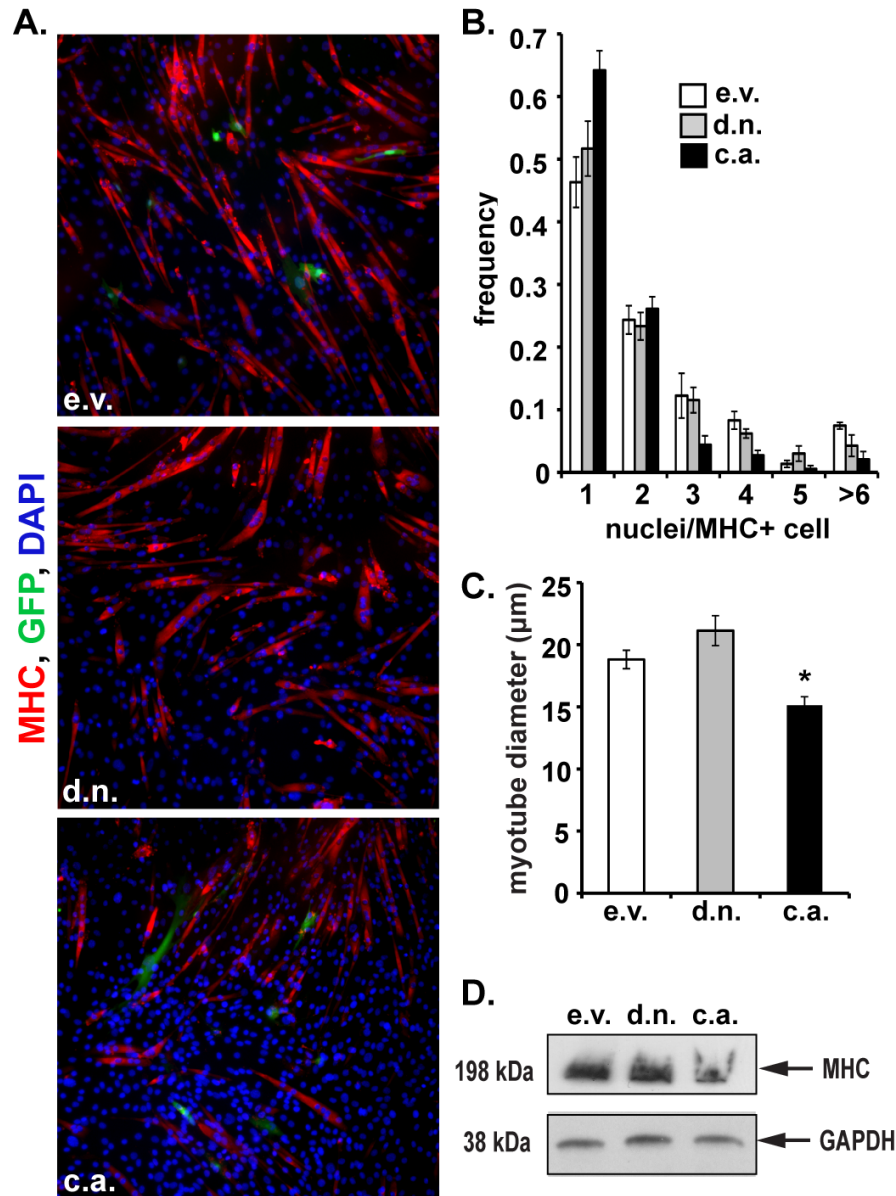


Figure 4.3: Increased NF- κ B activity in human primary pericytes (HPPs) inhibits myogenic differentiation of co-cultured myoblasts. A) Representative images of HPP/myoblast co-cultures for each of the IKK β conditions and the empty vector control following 96 h in co-culture under differentiation conditions. B) Distribution of nuclei for each of the IKK β conditions and the empty vector control following 96 h in co-culture with myoblasts under differentiation conditions. Data demonstrate the development of myotubes in each condition at 96 h. C) Myotube diameter for each of the IKK β conditions and the empty vector control following 96 h in co-culture under differentiation conditions. * signifies a significant difference ($P < 0.001$) from e.v. and d.n. transfected HPPs. D) Western blot of lysate from HPP/myoblast co-cultures, under each of the IKK β conditions and the empty vector control following 96 h in co-culture, probed for myosin heavy chain (MHC) and GAPDH as a loading control. All data are means \pm SEM.

Pericyte specific NF-kB activity non-cell-autonomously regulates myoblast proliferation:

In addition to affecting differentiation, it also became apparent during our image analysis that following 96 h of differentiation, our co-cultures with c.a. IKK β expressing HPPs had more cells, suggesting a possible effect on myoblast proliferation. This finding was confirmed by counting DAPI positive cells in each condition after 48 and 96 h of co-culture under differentiation conditions. We found no difference at 48 h, but a marked increase in cell number in the c.a. IKK β condition compared to the d.n. IKK β and e.v. conditions (Figure 4.4A and B). Because the number of GFP-positive HPPs declined over the 4 d differentiation period, the increase in cell number was likely due to proliferation of the co-cultured myoblasts.

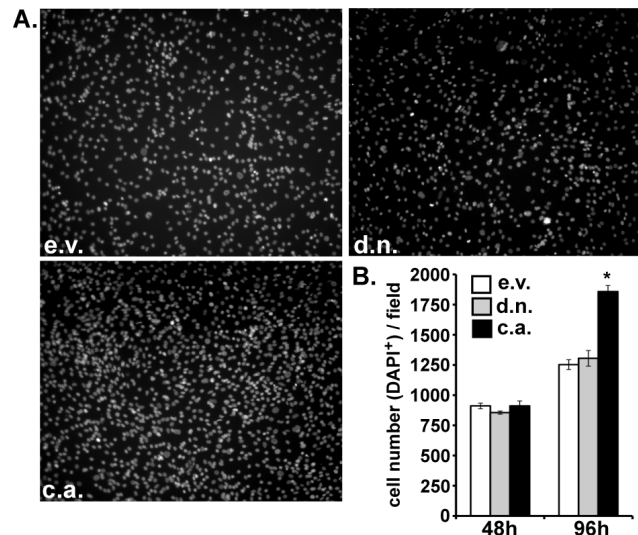


Figure 4.4: Increased NF-kB activity in human primary pericytes (HPPs) promotes proliferation of co-cultured myoblasts. A) Images of HPP/myoblast co-cultures for each of the IKK β conditions and the empty vector control, stained for nuclei following 96 h in co-culture. B) Cell number following 48 and 96 h of co-culture in each condition under differentiation conditions (DM). * signifies a significant difference (P<0.001) from e.v. and d.n. conditions at 96 h. All data are means \pm SEM.

Discussion

In vivo myogenesis requires a coordinated pattern of signaling crosstalk between multiple cell types that both reside in and infiltrate skeletal muscle during development and following injury (9, 21, 26). Previous work from our laboratory has indicated that pericyte-derived NF- κ B signaling is an important event in the early response to muscle injury in humans (18). We were therefore interested in whether pericyte specific NF- κ B activity was of consequence to the myogenic response shortly following muscle injury. To ascertain the effect of pericyte-specific NF- κ B signaling on myogenesis, we used an *in vitro* paradigm of myogenesis (C₂C₁₂ myoblasts under differentiation conditions) on to which we co-cultured human primary pericyte cells (HPPs). The major findings were that: 1) Inhibition of NF- κ B activation enhanced the myogenic potential of HPPs in a cell-autonomous manner and; 2) Constitutive activation of NF- κ B in HPPs non-cell-autonomously inhibited myogenic differentiation and promoted myoblast proliferation of co-cultured myoblasts.

NF- κ B affects the myogenic potential of HPPs

Numerous recent reports have suggested a possible role for perivascular-derived cells as non-satellite cell myogenic precursors, capable of spontaneous *in vitro* myogenesis and *in vivo* rescue of dystrophic rodent muscle (8, 11, 22, 23). In contrast to these studies, we were not able to detect spontaneous myogenesis in our HPP cultures over a 15 d period under a variety of culture conditions (i.e. conditioned and non-conditioned differentiation medium). However, when we cultured HPPs on to developing myotubes, we observed a

modest number of HPPs capable of myotube fusion, although that number was 3-fold lower than the number of myoblasts that were capable of fusion when co-cultured with developing myotubes at the same ratio. The relatively low efficiency of HPP myogenic fusion may in part be a function of the species difference between the developing myotubes (mouse) and the co-cultured pericytes (human). However, Gentile *et al.* (13) recently reported that human epicardium-derived cells fused to mouse myoblasts with high efficiency (12%) when co-cultured at similar ratios to the current study. In contrast, they reported that human adipose tissue-derived mesenchymal stromal cells fused less efficiently (<1%), suggesting that cell type, rather than species difference may more adequately explain myogenic potential. Nevertheless, our data are well supported by Kirilova *et al.* (19) who reported that a preparation of retina-derived perivascular cells from mouse likewise did not differentiate spontaneously, yet fused to developing myotubes *in vitro*. A possible explanation for the discrepancy of findings regarding spontaneous *in vitro* differentiation can perhaps be found in the source of the cells. Whereas both we and Kirilova *et al.* (19) used perivascular cells derived from vascular rich sources distinct from skeletal muscle (i.e. retina or placenta), others have used skeletal muscle-derived pericytes (11, 22). In this regard, skeletal muscle-derived pericytes may be more “conditioned” to adopt a myogenic phenotype. Alternatively, because these prior studies relied on flow cytometry-based cell sorting to isolate pericytes from skeletal muscle tissue, there may have been the possibility for satellite cell contamination.

Although we found that a relatively small proportion of HPPs were myogenic following 3 d of co-culture with myotubes, we did observe that inhibition of NF- κ B significantly increased their myogenic capability. This finding is consistent with the well-supported role for NF- κ B as a negative regulator of myogenesis (2, 3, 20). Interestingly, we did not find that constitutively active NF- κ B activity inhibited myogenic activity to a significantly greater extent than transfection with an empty vector control plasmid. This could be because we were underpowered in that we found so few instances of myogenic HPPs in either condition. Although we did note a lower total number of pericyte nuclei in the c.a. condition, there was no statistically significant difference between this condition and the empty vector control condition. However, notwithstanding the limited myogenic activity of HPPs, our data support the hypothesis that NF- κ B inhibition may represent a useful strategy to enhance the myogenic inclination of non-satellite cell muscle precursor cells, which may be important in optimizing current stem cell strategies for muscle degenerative disorders.

NF- κ B activation in HPPs affects co-cultured myoblast differentiation and proliferation

In a trophic-rich medium (GM), C₂C₁₂ myoblasts have the potential to proliferate indefinitely. However, when those growth factors are removed, as in the case of low serum culturing conditions, myoblasts will choose one of three developmental fates; A large portion of cells will upregulate myogenic regulatory transcription factors (e.g. MyoD and myogenin) and differentiate into multinucleated myotubes (25); A subset of cells will become quiescent as lineage restricted muscle stem cells (30); and finally, a

population of cells will not upregulate survival signals and die by apoptosis (10) (Figure 4.5). Our data suggest that HPPs with constitutively active NF- κ B, either via regulation of paracrine signaling or cell contact-mediated interactions, push local myoblasts to re-enter the cell cycle rather than to differentiate (Figure 4.5). Because HPP number declines over the 4 d differentiation period, yet the proliferative effect is most prominent at 4 d of co-culture, it is likely that HPPs exert this effect via paracrine signaling rather than cell contact-mediated mechanisms. In fact, several secreted cytokines/chemokines under NF- κ B transcriptional control have been shown to have pro-mitogenic and anti-differentiative effects on myoblasts. Some of these include monocyte chemoattractant protein-1 (MCP-1; CCL2) (27, 29), macrophage inflammatory protein-1 α (MIP-1 α ; CCL3) (15, 29), macrophage inflammatory protein-1 β (CCL4) (28, 29) and tumor necrosis factor- α (TNF- α) (1, 16). Specifically, the CC family chemokines (CCL2, CCL3, CCL4) were shown in a recent report to have a direct proliferative and anti-differentiative effect on local myoblasts, independent of their roles as inflammatory chemoattractants (29). Additionally, others have shown *in vivo* that vessel-associated endothelial cells are involved in the recruitment of inflammatory cells via CCL2 signaling following ischemic injury (24), suggesting that vasculature associated constituents, which are located in close proximity to satellite cells (6), provide this dual function. We have also shown, that 3 h following muscle damage in humans, that there is an increase in pericyte specific NF- κ B activity that is accompanied by robust transcriptional increases in the expression of CCL2 (17, 18), which is localized to satellite cells at 6 h post injury (17). Thus, the CC chemokines, specifically CCL2,

represent a group of strong candidates for NF- κ B dependent signals that may be responsible for our observed effects.

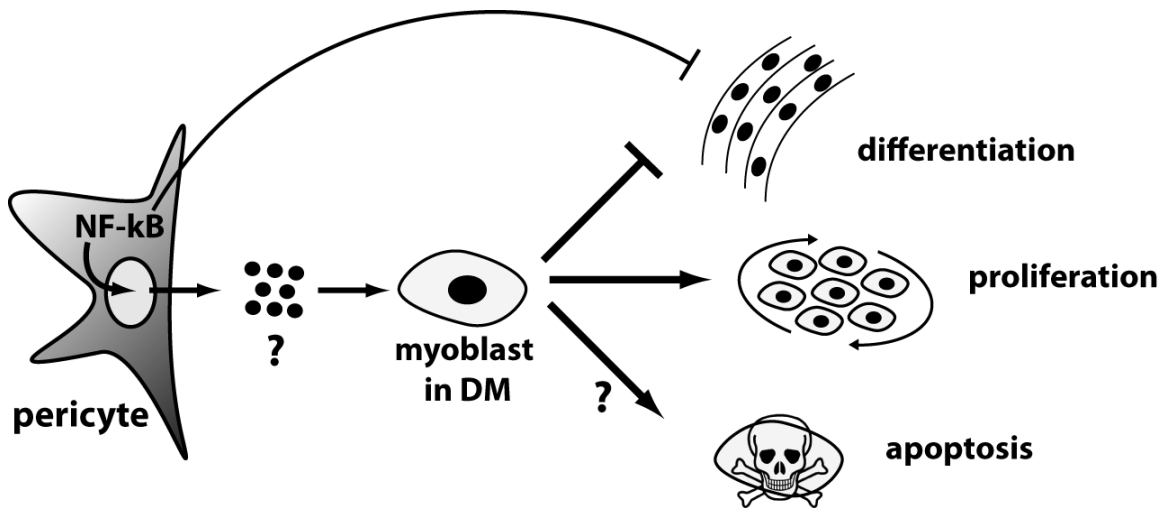


Figure 4.5: Model of cell- and non-cell-autonomous effects of pericyte-derived NF- κ B activation on local myoblasts under differentiation conditions: In low trophic factor medium (DM), myoblasts will choose one of three developmental fates; differentiation to multinucleated myotubes, quiescence or apoptosis. Activation of NF- κ B in co-cultured pericytes promotes myoblast proliferation *in lieu* of quiescence and inhibits myoblast differentiation, likely via paracrine signaling of an unknown secreted factor. It is currently unknown whether this event affects cell death of myoblasts in DM. Additionally, NF- κ B de-activation enhances myogenic differentiation of pericyte cells.

The model in Figure 4.5 depicts how pericyte-derived NF- κ B activity influences both intrinsic and extrinsic myogenic activity based on our data. While it is clear that non-satellite cell populations are capable of giving rise to myogenic-competent cells, and our data lends support to this notion, it is becoming widely-accepted that satellite cells are the primary source of muscle stem cells used for muscle growth and repair (5, 12). It is also evident that cell-cell contacts and extracellular signaling from the local niche play a role in myogenic activity of satellite cells (4, 21), yet this represents one of the most underexplored areas of satellite cell research (5). The spatial proximity of satellite cells to

the microvasculature has, for some time, been thought to foster interactions and facilitate systemic and local signaling to influence satellite cell activity (6). Our findings support this cross-talk relationship and furthermore, implicate NF- κ B signaling as a key mediator. This finding may have particular relevance for age-related skeletal muscle loss, which is characterized by a loss of regenerative capacity (14). Age-associated reductions in regenerative capacity seems to be fundamentally dependent on the local muscle environment, as satellite cells from old muscle regain their myogenic potential when transplanted into young muscle (7). Our data support a role for vascular-associated pericyte cells as important constituents of that regulatory environment.

Conclusion

In summary, We have provided additional supporting evidence for the myogenic capability of pericyte cells, although under our conditions, their contribution to *in vitro* myogenesis appears minor. We also demonstrate that, in addition to their potential as myogenic precursor cells, which has been the focus of much recent research, pericytes can function in a non-cell-autonomous manner to regulate myogenic activity of local muscle progenitors. Furthermore, we have determined that NF- κ B dependent signaling plays an integral role in both the autonomous and non-cell-autonomous myogenic contribution of pericytes.

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Driven primarily by the prospect of using stem cell therapy for the treatment of skeletal muscle loss associated with disease (i.e. Duchenne muscular dystrophy; DMD) and aging, there has been great interest in understanding the mechanisms of skeletal muscle myogenesis. Consequently, there has been a significant increase in the number of studies addressing these mechanisms in the recent years. However, a major limitation to our ability to translate this information to meaningful treatment outcomes is a paucity of data in relevant models of myogenesis in humans. While genetic animal models will continue to be valuable in unfolding the detailed molecular underpinnings of myogenesis, new data appear to suggest that there are fundamental differences between the human and rodent myogenic response (2). For example, virtually all of the work that has been done on satellite cell quiescence, activation and self-renewal has been performed with mouse satellite cells (6, 16), leaving doubt as to whether the same mechanisms are recapitulated in humans. Therefore, the overall objective of this dissertation was to characterize and identify early novel changes in molecular pathways and signaling in human muscle

following eccentric contraction-induced muscle damage, a stimulus known to result in a regenerative myogenic response.

To address this objective I used a translational “bedside to bench top approach.” That is, cellular and molecular observations were made in a relevant model of muscle regeneration in humans that were then used to ascertain potential mechanisms of action using *in vitro* methodology. Results from the human study (Chapter 3) provided novel evidence of NF- κ B activation in human muscle following muscle eccentric contractions that was localized primarily to muscle residing pericytes. Subsequent *in vitro* analyses (Chapter 4), using human primary pericytes (HPPs) with genetically induced over-activation of NF- κ B showed that NF- κ B functions intrinsically to inhibit myogenesis of HPPs and extrinsically to promote local myoblast proliferation and inhibit myoblast myogenic differentiation. ***Taken together, the fact that NF- κ B activation (3 h) precedes the early proliferative response of satellite cells (Figure 2.1), suggests that pericyte-specific NF- κ B activation may act to promote satellite cell proliferation and inhibit myogenic differentiation in the early hours following muscle injury.*** Proliferation of satellite cells following injury has been shown to be essential for muscle regeneration (Figure 2.2), yet the signals that regulate this critical event are largely unknown. This early pro-proliferative/anti-myogenic response is critical to ensure that the normally quiescent satellite cell pool is sufficient for both muscle repair and niche maintenance. As detailed in the discussion of Chapter 4, it is likely that this NF- κ B dependent regulatory mechanism is mediated by paracrine signaling of chemokines, which may additionally

play a dual role in recruiting inflammatory molecules (15). A schematic of this proposed model, based on data from this dissertation is provided in Figure 5.

Future Directions

I have speculated that the non-cell-autonomous effects of pericyte-derived NF- κ B activity on myoblast myogenesis is mediated by paracrine signaling (Chapter 4). Alternatively, cell-cell interactions could have produced the observed effects, although this is unlikely due to low pericyte/myoblast ratios that exist at later time points. Nevertheless, it will be important for future work to determine the exact mechanism of action via similar trans-well experiments conducted by Mathew *et al.* (8). If in fact paracrine signaling underlies the non-cell-autonomous effects of NF- κ B activation in pericytes, the identification of the secreted factors under NF- κ B control would be important in further defining the environmental signals that are critical for effective muscle regeneration. Dahlman *et al.* (4) have recently provided an exemplary pattern for the elucidation of paracrine signaling in muscle resident cells. They observed that NF- κ B activation in muscle resident fibroblasts was critical for proper muscle development in mouse neonates. Using mouse genetic models they were able to show that NF- κ B dependent expression of nitric oxide synthase (iNOS) was responsible for the observed non-cell-autonomous effects of fibroblasts on local satellite cells. Certainly, a similar pattern of experiments could be conducted using a candidate gene approach; for example, CCL2 (as discussed earlier) or iNOS. Alternatively, genetic screening via microarray could be used to identify highly

expressed transcripts for secreted factors in pericytes with altered NF- κ B activity. This type of analysis could then be followed up using mouse genetic and *in vitro* models to determine their effect on local myoblast proliferation and differentiation.

This dissertation has also provided evidence confirming the myogenic potential of pericytes and the involvement of NF- κ B in that developmental decision (Chapter 4). Several groups have reported that using muscle and non-muscle derived pericytes is a viable stem cell treatment strategy in models of DMD (3, 5, 10, 11). In each case, pericytes have been used successfully to restore dystrophin expression in the dystrophin-null *mdx* mouse, yet the extent of rescue has been limited to the local area of intra-muscular pericyte injections. However, in one case (10), pericytes were mildly effective at restoring dystrophin expression when delivered systemically through the vasculature. In this regard, pericytes hold promise as potentially effective stem cells for the treatment of DMD, as they have been shown to effectively move out of the vasculature and into skeletal muscle (12). Data from this dissertation have shown that inhibition of the NF- κ B signaling pathway (by only approximately 50%) significantly increases myogenic potential of pericyte cells. Therefore, it is likely that inhibition of NF- κ B may be a viable strategy to increase the myogenic efficiency of pericyte cells in the treatment of DMD. Additionally, it is unknown whether pericytes contribute *in vivo* to muscle repair following injury. Although it is generally believed that satellite cells are the principle, if not sole, progenitors responsible for the repair of injured muscle (6), no study has yet looked at the contribution of pericytes to muscle repair. Unlike bone marrow-derived hematopoietic cells, which have also been studied extensively as a potential myogenic-

competent stem cell (7, 9, 12), pericytes are natively found in skeletal muscle and, in fact, constitute approximately 7% of all nuclei found in human skeletal muscle cross sections (Chapter 3). Their proximity to skeletal muscle therefore makes them well suited to the role of skeletal muscle repair.

It is well established that muscle damage is accompanied by a predictable and well-characterized infiltration of inflammatory cells (14). Furthermore, the infiltration of inflammatory cells has been shown to be essential for normal, effective regeneration (1, 13). In the Chapter 4 discussion, I also speculate that pericytes contribute to the recruitment of inflammatory cells following muscle damage. Data from this dissertation show clearly that pericytes respond to muscle damage by altering activation of a major signaling pathway associated with inflammation (NF- κ B) and the recruitment of monocytes. It is therefore possible that pericytes play a dual role (as described schematically in Figure 5) in the secretion of NF- κ B dependent paracrine factors to affect satellite cell activation and proliferation, as well as the recruitment of inflammatory cells from the circulation. Given their proximity to both satellite cells and the vasculature, this dual role is certainly worth further exploration. In conclusion, the data presented in this dissertation will pave the way for studies to further explore the interactive role of pericytes and skeletal muscle in muscle regeneration.

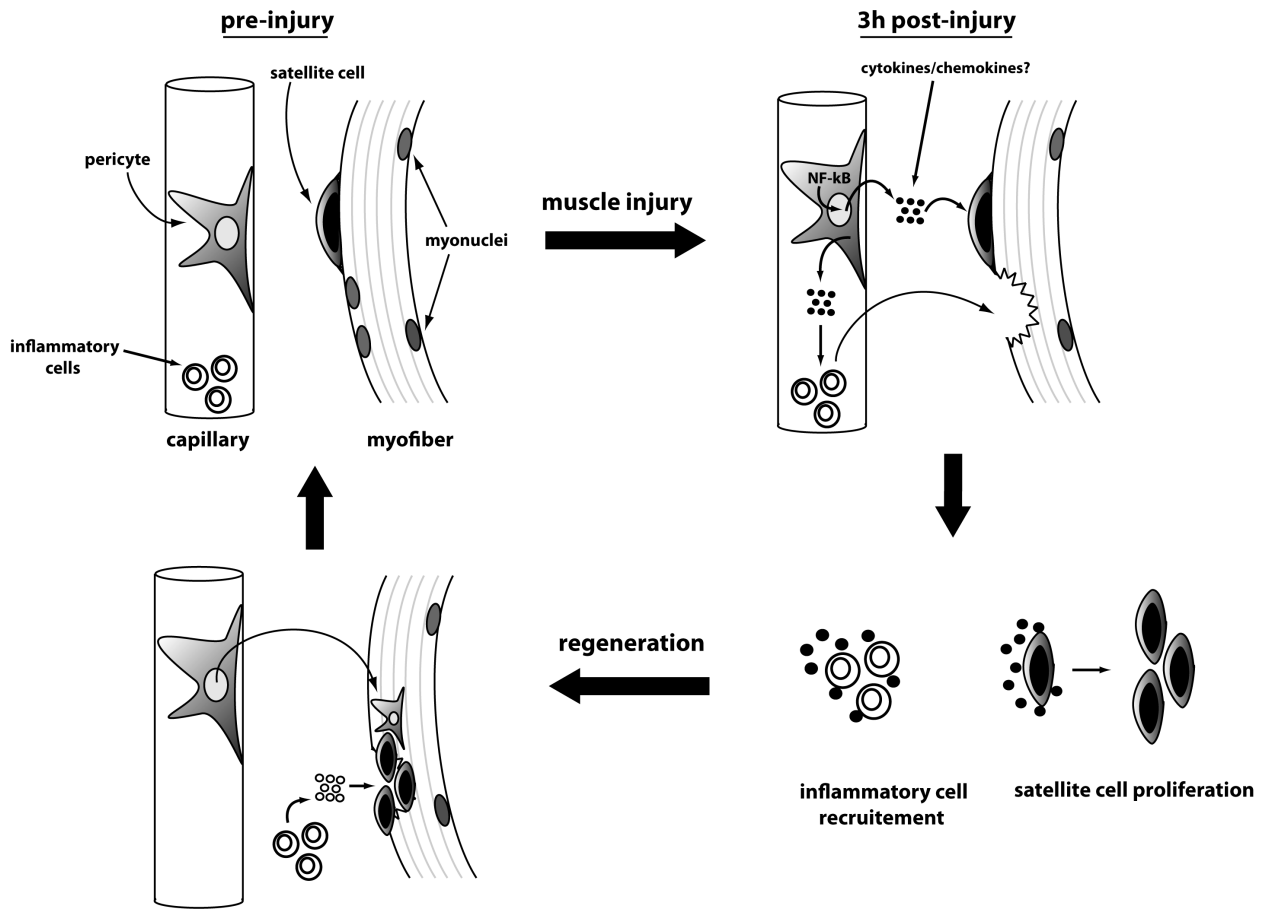


Figure 5.1: Proposed model of molecular and cellular signaling following muscle injury in humans, based on data from this dissertation and published research. Shortly following muscle injury, NF- κ B is activated in muscle residing pericytes. Activation of NF- κ B results in secretion of cytokines/chemokines, which promote the proliferation of satellite cells. Additionally, these cytokines/chemokines may play a role in recruiting inflammatory cells. The expanded satellite cell pool then migrates to the site of damage and fuses to repair the fiber. Infiltrating inflammatory cells provide further signaling input to promote proliferation and differentiation of satellite cells. Additionally, pericytes may contribute to myofiber repair by fusing to the damaged fiber during later phases of regeneration.

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APPENDIX

KNOCKDOWN OF METALLOTHIONEIN 1 AND 2 DOES NOT AFFECT ATROPHY OR OXIDANT ACTIVITY IN A NOVEL *IN VITRO* MODEL

The work for this study was done just prior to the previously discussed dissertation studies and serves as a proof of concept for how *in vitro* models can be used effectively to elucidate the mechanistic relationships of observations made in humans. In addition to providing novel data on the role of metallothioneins during muscle atrophy, this study also set the stage for my dissertation studies by providing invaluable experience using *in vitro* models and inducible gene expression paradigms. It was published in the Journal of Applied Physiology in November 2010.

Hyldahl RD, O'Fallon KS, Schwartz LM, Clarkson PM. Knockdown of metallothionein 1 and 2 does not affect atrophy or oxidant activity in a novel *in vitro* model. *J Appl Physiol*, Nov 2010; 109: 1515-1523.

Introduction

Skeletal muscle atrophy occurs from a myriad of distinct stimuli. Conditions generally resulting in atrophy include immobility, disuse, denervation, or as the consequences of certain chronic disorders such as sarcopenia, cancer, AIDS and diabetes (28-32). Muscle atrophy is commonly characterized by a loss of balance between protein synthesis and degradation that ultimately results in a net loss of contractile proteins (17, 33). Atrophy

results in decreased muscle fiber size, force production and fatigue resistance, which in turn can lead to weakness, instability and a decreased quality of life.

Muscle atrophy can be mediated by a number of distinct signal transduction pathways. As examples, disease-related atrophy is brought about by elevated levels of circulating cytokines and/or glucocorticoids (34-36), while disuse atrophy appears to be triggered by intracellular signaling events as a result of decreased muscular tension (13). Muscles that no longer bear weight or are immobilized for extended periods of time initiate pathways leading to protein loss, and some of these pathways have been well characterized (4, 37). However, potential upstream molecules regulating these processes are still poorly defined.

It has been extensively documented that expression of metallothioneins (MT) are significantly upregulated in muscle cells following an atrophy stimulus in both humans and animal models (32, 38-40). MTs are ubiquitously expressed proteins with a diversity of reported intracellular functions (41). In rodents, there are 4 known isoforms of MT (1-4), two of which (MT1 and 2) are expressed in skeletal muscle. In humans, at least 17 isoforms have been identified. Different isoforms of MT are distributed in a wide variety of tissue, and for the most part, appear to have redundant functions although some specialization has been noted (42). While it has been difficult to define a consensus biological function for MTs, it is clear that they play important roles in both binding of heavy metals and as endogenous antioxidants (43). More recently, their role as

antioxidants and their ability to protect against oxidative damage in various tissues has been elucidated (44).

As part of our ongoing studies to define the molecular mechanisms that mediate skeletal muscle atrophy in humans, we have performed microarray analysis with mRNA isolated from muscle biopsies 48 hours after knee joint immobilization, unilateral lower limb suspension and spinal cord injury (32, 38) (K Sewright-Reich, unpublished observations). This unique set of data, collected at the same time point relative to the experimental manipulation or injury for three distinct conditions, has permitted a detailed comparative analysis to ascertain a possible common program for gene expression following disuse. One of the few gene clusters to be commonly increased following all 3 forms of disuse was the MTs. These observations are consistent with data from animal models, where a significant increase in MT mRNA has been measured following a diverse range of atrophy-inducing manipulations (31, 39, 45). Despite the preponderance of gene expression data indicating increased expression levels of MTs following an atrophy stimulus, the potential role(s) for MT in the muscle atrophy program have not been identified. Given their ability to scavenge free radicals and protect against oxidative injury (46), it has been suggested that increases in MT expression may be an initial response to offset increases in oxidative stress associated with a muscle atrophy stimulus.

In the current study, we have employed the use of an *in vitro* model of muscle atrophy to test the hypothesis that transient MT gene knockdown would result in greater oxidative stress and an enhanced response to an atrophy stimulus. Given the inconsistencies of

current modes of atrophy induction in C₂C₁₂ myotubes (47, 48) and their requirement for exogenous factors not normally associated with disuse atrophy, we have also sought to develop and characterize a novel *in vitro* model of muscle atrophy to test our hypothesis. Herein we describe a simple and reliable method for the induction of atrophy in culture myotubes and subsequently apply this model to more clearly delineate the role of MTs during muscle atrophy.

Methodology

Cell Culture to Induce Myotube Atrophy: C₂C₁₂ myoblasts were obtained from the American Type Culture Collection (ATCC) and expanded and frozen down. Only early passage cells were used in these studies. For experiments, cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals Norcross, GA), to create a growth factor rich growth medium (GM) at 37°C in 10% CO₂. At 80-90% confluence, cells were transferred to differentiation medium (DM) (DMEM plus 2% horse serum (Hyclone, Logan, UT)) to induce myotube formation. Following 4 days in DM, fused myotubes were re-introduced into the high serum GM for 48 hours to produce a hypertrophic response (Figure A.1). Experimental cells were then switched back to DM for a maximum of 72 hours to produce the desired atrophic response (serum reduction (SR)). Control cells remained in GM (See Figure A.1). Data were collected at baseline, 6, 12, 24, 48 and 72 hours under the growth factor reduced and control conditions.

siRNA: All siRNA duplexes were obtained from IDT (Coralville, IA) and used at a final concentration of 10nM for knockdown experiments. The sequences for the MT1 and MT2 siRNA duplexes were as follows: MT1, 5'-AGCGUCUUCCUAUACAGUCCACCC-3' and MT2, 5'GUAAAUAGACCAUGUAGAAGCCUAG-3'. A standard non-silencing, scrambled control siRNA duplex was obtained from IDT (Coralville, IA).

Transfections: Following 4 days of differentiation (the onset of GM re-introduction), myotubes were transfected in either 24-well plates, 6-well plates or slide flasks with 10nM MT1, MT2 or scrambled control (non-silencing) siRNA nucleotides using Lipofectamine RNAi max (Invitrogen, Carlsbad, CA). This time point was chosen so that MT1 and MT2 expression would be reduced at the onset of DM re-introduction (0h). Both Lipofectamine and the appropriate siRNA sequence(s) were first diluted individually in Opti-MEM I Reduced Serum medium (Invitrogen, Carlsbad, CA). The diluted Lipofectamine and siRNA sequence(s) were then mixed and allowed to complex for 20 minutes at room temperature. Lipid/siRNA complexes were added to each well at a concentration of 10nM in an appropriate volume for each plate size and incubated for 24 hours. After 24 hours, transfection medium was removed and replaced with GM. A scrambled, non-silencing sequence was transfected into parallel wells for all experiments. To determine transfection efficiency and siRNA stability, parallel cultures were also transfected with TYE-563 labeled double stranded control sequences (IDT, Coralville, IA) under the same conditions. Myotubes were then visualized and photographed using a Nikon model TMS inverted fluorescence microscope and a SPOT Insight imaging color

camera (Diagnostic Instruments, Sterling Heights, MI). Efficiency of knockdown was also determined on the RNA level with quantitative real-time PCR (qRT-PCR) and on the protein expression level using Western blot analysis.

Immunocytochemistry: To assess changes in myotube morphology, myoblasts were grown in chamber slide flasks and treated as above. At each time point, myotubes were fixed for 2 minutes in 2% paraformaldehyde at room temperature. The myotubes were then permeabilized for 3 minutes with Karsenti's Lysis Buffer (0.5% Triton X-100, 80mM PIPES, 1.0 mM MgSO₄, 5.0 mM EGTA, pH 7.0), after which they were rinsed twice with PBST (PBS, 0.1% Tween-20). Myotubes were then incubated with the mouse monoclonal MF20 antibody against myosin heavy chain (Developmental Hybridoma Bank, Iowa City, IA) for 60 minutes in the dark at room temperature. After 3 washes with PBST, the myotubes were incubated with AlexaFluor 488 anti-mouse (Invitrogen, Carlsbad, CA) for 60 minutes in the dark. Myotubes were washed again 3 times in PBST and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). Samples were visualized and measured as described below.

Myotube Morphology Analysis: Myotubes were visualized and photographed with a 20X objective on a Nikon model TE-2000 inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI). The diameters of the myotubes were quantified using Metamorph Imaging software (Molecular Devices, Downingtown, PA) at 24, 48 and 72 hour time points under all experimental and control conditions. Approximately 100 diameters were randomly selected from 12 representative

microscope fields in each condition from 3 independent experiments. Three lines running perpendicular to the orientation of the fixed myotubes were drawn at evenly spaced intervals across each field using the Metamorph software. Diameter measurements were made in micrometers at all points where the line crossed a myosin heavy chain (MyHC) positive myotube.

Total RNA, DNA, and qRT-PCR: Plates were scraped and the myotubes separated from the residual mononucleated myoblasts by filtering through a 50 μm sieve. Verification of the separation was made via phase contrast microscopy. Total RNA was extracted from myotubes at 6, 12, 24 and 48 hours following SR using TriZol reagent (Invitrogen Carlsbad, CA) according the manufacturer's instructions. RNA concentration was determined by optical density on the Nanodrop ND-100 spectrophotometer (Nanodrop Products, Willmington, DE). Total RNA was reverse transcribed using a cloned murine leukemia virus reverse transcriptase (Fermentas, Glen Burnie, MD) according to the manufacturer's instructions. The relative levels of MT1, MT2, Atrogin1 and MuRF1 mRNAs were determined by quantitative real-time PCR (qRT-PCR). ABgene Absolute qPCR SYBR Green Master Mix (ABgene, Surrey, UK) with ROX dye was used for all PCR protocols. qRT-PCR reactions were performed in 96-well plates with all cDNA samples from each time point for both experimental and control myotubes run in triplicate for each gene of interest. The average cycle threshold (Ct) value for triplicate samples was used for data analysis. Samples were run for 40 cycles of amplification on a MX3000p Real-Time PCR System (Stratagene, La Jolla, CA). At the end of each reaction, a melting curve analysis was run to ensure target specificity. Differences in gene

expression were determined by the delta Ct relative quantification method. Values were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an internal reference gene whose expression did not change in response to the treatment (data not shown). Forward and reverse primers (IDT, Coralville, IA) for all genes of interest (see Table A.1 for sequences) were designed using NCBI gene sequences with the Primer Express program v 2.0 (Applied Biosystems, CA). All primers were tested for efficiency via a standard curve and demonstrated efficiencies between 95 and 104%.

Table A.1: Primer sequences

Gene Name	Forward Primer	Reverse Primer
Atrogin 1	5' - AGGCTTCTAATTGGATGGCTGGGA - 3'	5' - CCCFCAGTTTCAAGCCAATGCTTA - 3'
MuRF 1	5' - TCCGAGTGGGTTTGGAGACAAAGA - 3'	5' - AGGCTTGGTAAACATCTCCAGGCA - 3'
MT 1	5' - ACCACGACTTCAACGTCCTGAGTA - 3'	5' - AGGAGGTGCACTTGCAGTTCTTG - 3'
MT 2	5' - TCTGCAAAGAGGCTTCCGACAAGT - 3'	5' - TTGTGGAGAACGAGTCAGGGTTGT - 3'
GAPDH	5' - AGAACATCATCCCTGCATCC - 3'	5' - ACACATTGGGGGTAGGAACA - 3'

Western Blotting and Antibodies: C2C12 myotubes were isolated as above, and incubated in lysis buffer (20mM Tris pH7.5, 200mM NaCl, 0.25% Np-40, 1mM PMSF, 1mM sodium orthovanadate, 10mM NaF) on ice for 1h. The cell lysate was then centrifuged at 10,000xg for 10 minutes and the supernatant was removed. Protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) was then added to all samples. Samples were analyzed for total protein concentration using the BCA Assay (Pierce, Rockford, IL). The

samples were boiled for 5 minutes and equal amounts of protein were loaded into 4-15% gradient sodium dodecyl sulfate (SDS) poly-acrylamide gels (Bio-Rad Laboratories) and separated by electrophoresis (60-90min@100v). Fractionated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), which was then incubated with the primary antibody in 5% BSA in 1XPBST with rocking motion overnight at 4 degrees C. After washing in PBS supplemented with 0.5% tween-20, the membrane was incubated with an HRP-labeled secondary antibody. Membranes were then treated with enhanced chemiluminescence (ECL) for two minutes, wrapped in plastic film, and exposed to Kodak film. Bands from each blot were quantified via densitometry using ImageJ software (NIH). The antibodies used include: rabbit polyclonal anti-GAPDH (1:5000; Abcam, Cambridge, MA); mouse anti-myosin heavy chain monoclonal antibody MF20 (1:100, Developmental Hybridoma Bank, Iowa City, IA); rabbit polyclonal anti-phospho-AKT (ser473) (1:500, Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-metallothionein antibody (which recognizes all MT isoforms) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); and anti-mouse and anti-rabbit HRP-conjugated secondary antibodies (1:1000, Bio-Rad Laboratories, Hercules, CA).

Assessment of Intracellular Oxidant Activity: A 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein, diacetate, acetyl ester (CM-H₂DCFDA) (Invitrogen, Carlsbad, CA) assay was used to measure intracellular oxidant activity and provided a non-specific measure of oxidative stress. The chemically reduced parent compound (CM-H₂DCFDA) is non-fluorescent until the acetate groups are removed by intracellular

esterases. Oxidation by radical species occurs within the cell and forms fluorescent dichlorofluorescein (DCF). Fresh stock solutions of CM-H₂DCFDA were reconstituted in cell culture grade DMSO (ATCC) and diluted in 1XPBS ($\leq 0.01\%$ vol/vol PBS) immediately prior to each experiment. Extracellular hydrolysis of CM-H₂DCFDA was minimized using PBS as a loading buffer. After dye loading, a ~15 minute recovery period was given to allow cellular esterases to hydrolyze the acetate groups on the dye that prevent oxidation. This process renders the dye responsive to oxidation by free radicals. All experiments were conducted in darkness to reduce inadvertent photo bleaching. Prior to experimental manipulations, myotubes cultured in sterile 96 well plates were washed once with PBS and incubated in loading buffer (PBS supplemented with 10 μ M CM-H₂DCFDA) for ~30 minutes at 37°C. Cells were then washed twice with PBS and incubated for ~15 minutes (recovery period) prior to returning myotubes to their corresponding experimental culture medium (serum reduced or control). DCF fluorescence was measured at 37°C on a fluorescence multiwell microplate reader (FLUOstar Optima, BMG LABTECH) at excitation and emission wavelengths of 485nm and 538nm, respectively. Baseline DCF fluorescence was measured immediately prior to SR and at 1, 2, 4, 6, 12, 24, and 48 hours post-SR. Background auto-fluorescence of DCF (measured from both DM and GM containing myotube-free 8 well lanes of each 96 well dish) was subtracted from negative controls and the corresponding experimental or control myotubes. Net intracellular oxidant activity in the serum-reduced conditions was expressed as the percentage of DCF fluorescence in the control condition (high serum). The rate of change in intracellular oxidant production was calculated as the average slope of the normalized values between time points.

Statistical Analysis: Data are presented as means \pm SE. All qRT-PCR, morphology, Western blot and DCF data were analyzed using a two-way analysis of variance (ANOVA) with factors for treatment and time. Where there was a significant effect, Tukey's Honest Significant Difference test was applied. All statistical analyses were computed using a SAS statistical software package. Significance was set a priori at $p < 0.05$.

Results

Development of an in vitro model for skeletal muscle hypertrophy and atrophy. To examine the role(s) of metallothioneins in muscle atrophy, we needed to develop an appropriate *in vitro* model. After several experimental paradigms, we selected the one outlined in Figure A.1. Briefly, we differentiated C₂C₁₂ myoblasts into myotubes by changing the cells from a growth factor rich growth medium (GM) to a differentiation medium (DM) that was deficient in these factors. After 4 days in DM, myotubes were returned to GM for two more days. Control cultures remained in GM for the subsequent two days while test cultures (referred to as "SR" for serum reduced) were returned to DM for the same period of time.

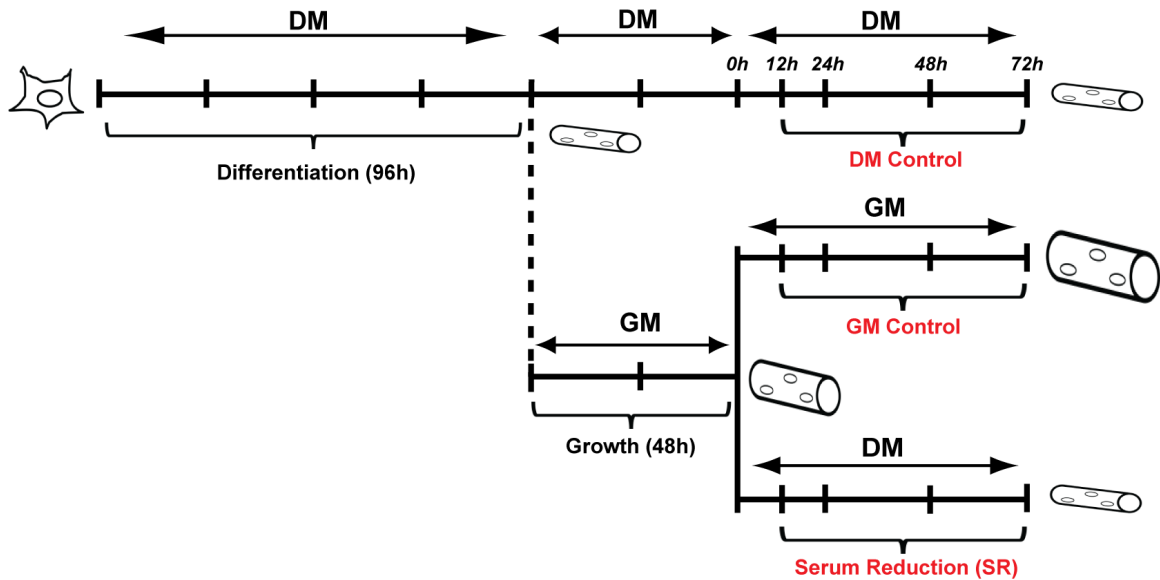


Figure A.1: Schematic drawing depicting the serum reduction (SR) paradigm used to produce myotube atrophy and for testing the effect of metallothionein knockdown. C_2C_{12} myoblasts were differentiated for 4 days in low serum differentiation medium (DM) followed by a 2-day period of myotube growth in high serum growth medium (GM). Control cells remained in GM following the 2-day growth period and SR myotubes were put again in low serum medium (SR) to induce the desired atrophic response. The specific analyses at each time point are detailed in the methods.

We then tested the hypothesis that SR results in myotube atrophy as measured by myotube diameter and protein loss. Myotube diameter was found to be significantly decreased in SR cultures relative to the GM control cells at 24, 48 and 72 hours ($p < 0.01$) (Figure A.2A and B). Additionally, myotube diameter was significantly different at all time points under the SR condition compared to baseline (0h) levels ($p < 0.05$). Myotube diameter was expressed in micrometers, rather than as a percentage of control to demonstrate that significant decreases in size were being driven by SR rather than an increase in size of the control myotubes. Further confirmation of morphological changes following SR was assessed by changes in the protein expression of the sarcomeric protein

myosin heavy chain (MHC). We found that 24, 48 and 72 hours of SR was effective at reducing MHC levels relative to control cells ($p < 0.01$) and over time compared to the baseline level ($p < 0.01$) (Figure A.2C and D). GAPDH was used as a loading control for the Western blots.

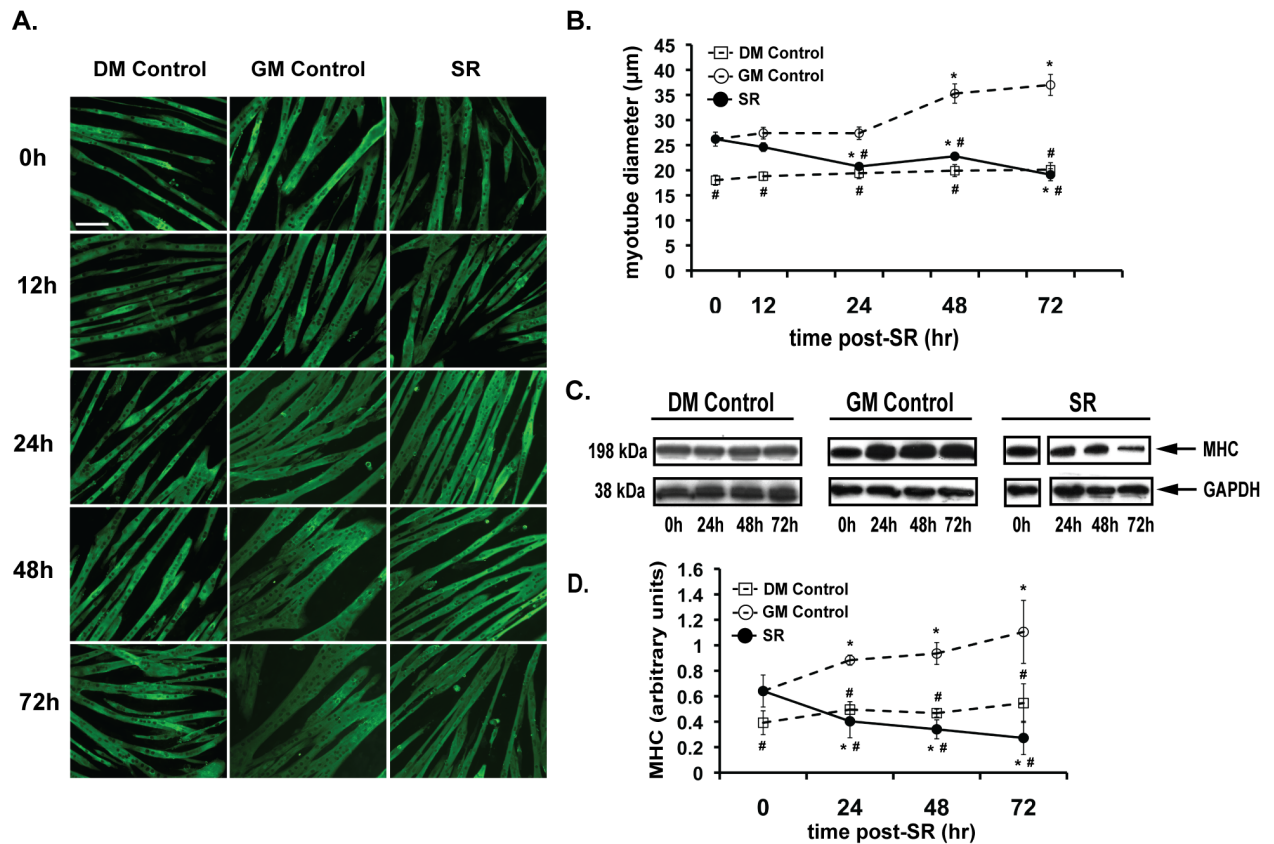


Figure A.2: Serum Reduction (SR) reduces myotube diameter and myosin heavy chain (MHC) protein content. A) Representative images from control and SR myotubes at 24, 48 and 72 hours stained with an antibody against MHC. The unlabeled dots are myonuclei. Scale bar equals 100µm. B) Myotube diameter of control and SR myotubes at 24, 48 and 72 hours; data are expressed as mean diameter in micrometers plus and minus the SEM. Data are from 4 independent experiments. * = significant differences from control myotubes ($p < 0.01$), ‡ indicates significant differences from myotubes at baseline (0h) ($p < 0.05$). C) Representative Western blots of MHC and GAPDH (loading control) expression from control and SR myotubes at 24, 48 and 72 hours. D) MHC protein in SR treated myotubes from 4 independent experiments expressed as a percentage of control. * = significant differences from control myotubes ($p < 0.01$), ‡ indicates significant differences from myotubes at baseline (0h) ($p < 0.01$).

SR-mediated changes in protein synthesis/degradation pathway components. To further characterize the SR model of atrophy, we used Western blot analysis to monitor the expression of several key regulatory proteins. Phosphorylation of the serine/threonine kinase AKT is associated with cell growth (4). The levels of AKT phosphorylation at ser473 significantly declined over time following transfer to SR (Figure A.3A and B). In contrast, transfer of myotubes into GM resulted in AKT phosphorylation at 12h and 72h. SR treatment also led to increased expression of the atrophy associated ubiquitin E3 ligases atrogenin1 and MuRF1 at 48 hours by 1.8 and 2.1 fold, respectively, when normalized to control cells ($p < 0.05$) (Figure A.3C).

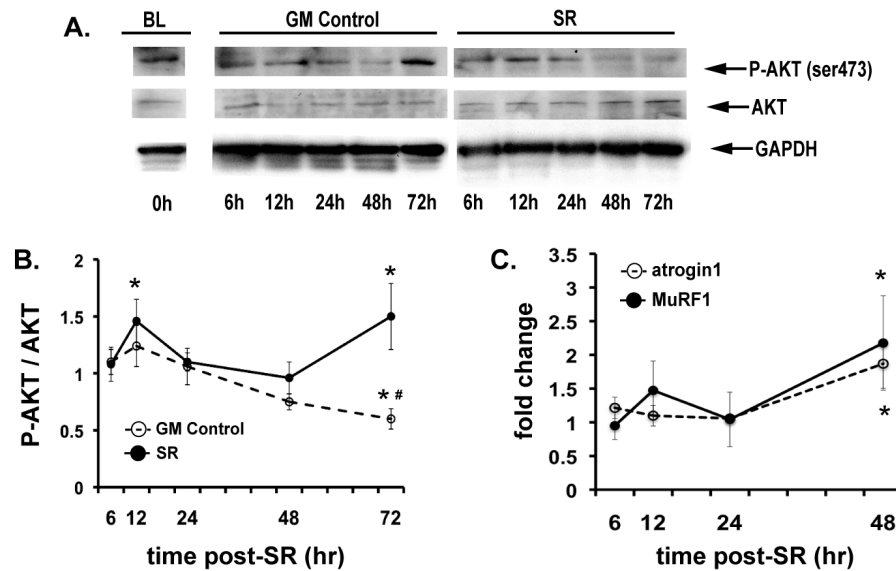


Figure A.3: Decreased activation of AKT and increased expression of the ubiquitin E3 ligases atrogenin1 and MuRF1 in myotubes in response to SR treatment. A) Representative immunoblot of phosphorylated AKT (ser473), total AKT and GAPDH over time in control and SR myotubes. B) Densitometry measurements of the phosphorylation state of AKT in SR treated myotubes from 3 independent experiments, expressed as p-AKT/AKT. * = significant differences from control myotubes ($p < 0.01$), ‡ indicates difference from myotubes at baseline. C) qRT-PCR analysis of atrogenin1 and MuRF1 mRNA expression over time in SR treated myotubes from 4 independent experiments. Data are presented as fold change relative to control myotubes. Dashed line indicates control myotube level, or no change. * = significant difference from control myotubes ($p < 0.05$).

Metallothionein expression in SR and siRNA transfected myotubes. To determine if MT is induced in our SR model similarly to other atrophy models, we measured endogenous MT1 and MT2 expression at various time points following SR. Expression of MT1 and MT2 mRNA increased by 2.3 and 2.6 fold, respectively, at 6 hours post-SR compared to control myotubes (Figure A.4A). There were no other significant differences in expression of either gene at any other measured time point. Both genes also tended to follow a very similar pattern of expression.

We next designed siRNA sequences that would selectively target MT1 and MT2 transcripts. Following co-transfection of myotubes with these siRNAs 48 hours prior to SR, we observed that there were 78% and 69% reductions in the basal levels of MT1 and MT2 mRNA, respectively at the 0 hour time point (beginning of the SR stimulus). At 24h post-SR (Figure A.4B), there were 82% and 69% for MT1 and MT2, respectively. Western blot analysis was used to verify that decreases in MT mRNA resulted in decreases in protein expression. At 48 hours post-MT1 and MT2 siRNA co-transfection (0h), there was a notable reduction in MT protein (Figure A.4C).

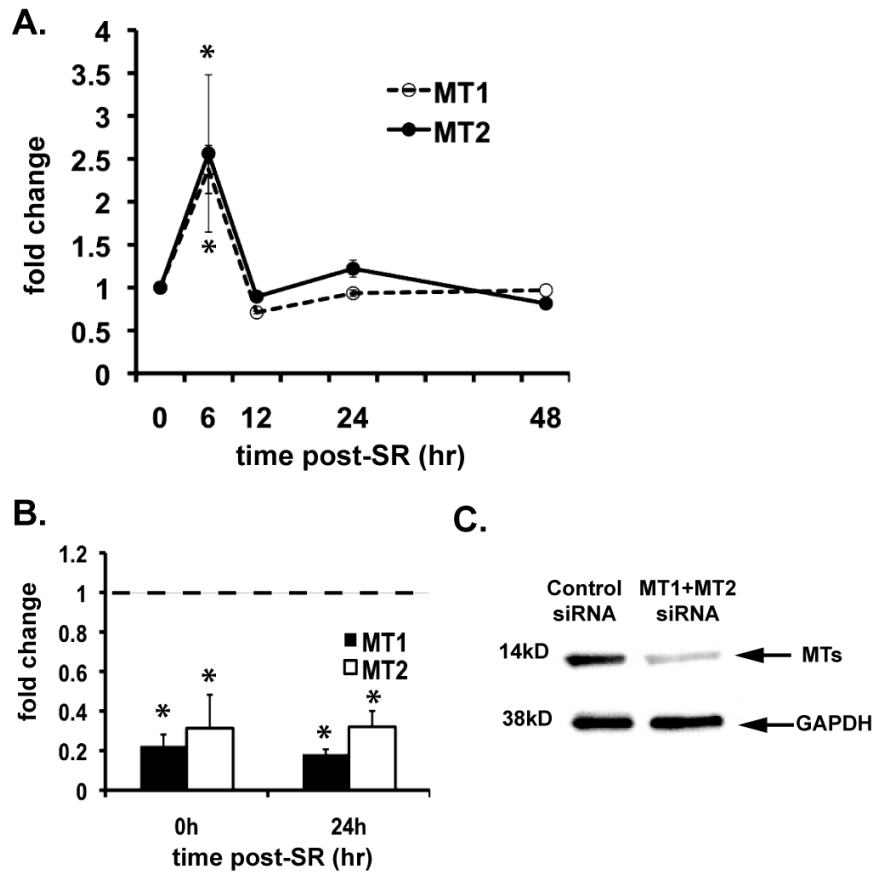


Figure A.4: MT expression in control and siRNA transfected myotubes in control and SR treated cells. A) Endogenous MT1 and MT2 expression in SR treated myotubes compared to control myotubes from 3 independent experiments. * = significant differences from control myotubes ($p < 0.01$) B) Expression of MT1 and MT2 at baseline (0h) and 24 hours post-SR in myotubes that were transfected 48 hours prior with siRNA duplexes targeted at MT1 and MT2. C) Immunoblot using an antibody that recognizes all MT isoforms, 48 hours following transfection (0h) with either siRNA targeted at MT1 and MT2 or a scrambled control siRNA sequence.

Oxidant activity in SR and siRNA transfected myotubes. One of the more well characterized roles for MTs in cells is to quench free radical levels (44). To measure intracellular oxidant activity in myotubes, we took advantage of the dichlorofluorescin (DCF) assay. Myotubes were pre-loaded with DCF and then fluorescence intensity was measured at 0, 6, 12, 24 and 48 hours post-SR. SR resulted in a significant increase in

oxidant activity over time compared to control myotubes ($p < 0.01$) (Figure A.5A and B), with the greatest changes occurring at the early time points between 6 and 12h post-SR (Figure A.5B). Knock-down of MT expression with siRNAs did not significantly reduce SR-induced increases in oxidant activity (Figure A.5).

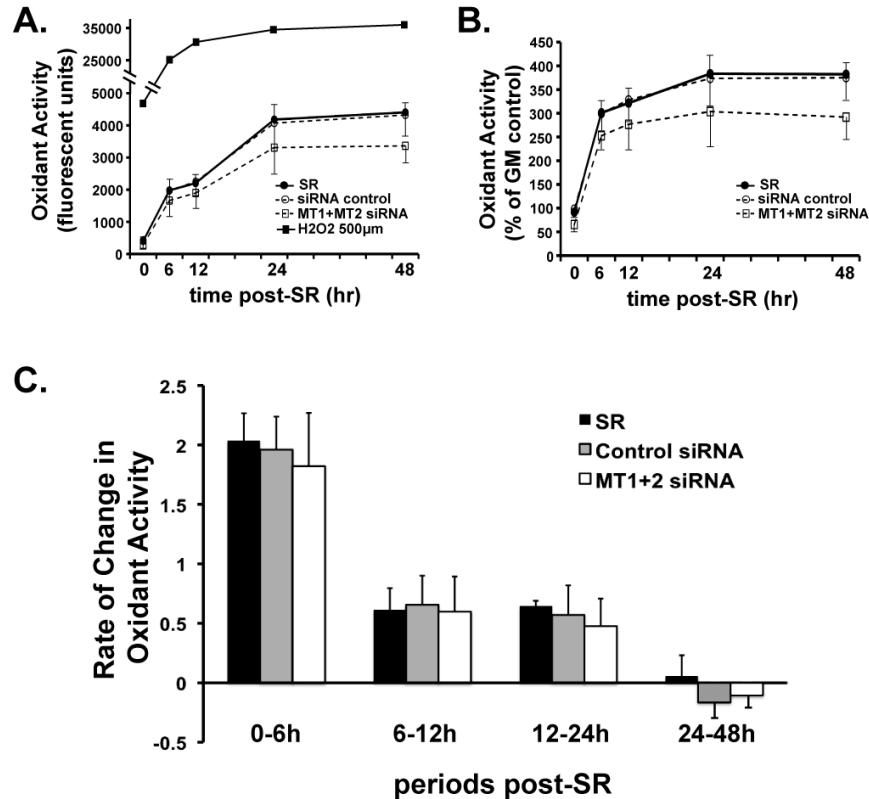


Figure A.5: Intracellular oxidant activity increases following SR, but is not affected by MT1 and MT2 knockdown. A) Oxidant activity normalized to control myotubes over time from 3 independent experiments. B) The rate of change in oxidative activity development following SR for each time period over the 2-day experiment, calculated by the slope of the line between the two time points.

Myotube diameter in siRNA transfected myotubes. To determine the effect of MT1 and MT2 knockdown on the development of muscle atrophy, we co-transfected myotubes with an siRNA sequence specific for MT1 and MT2 or a control, non-silencing siRNA 48

hours prior to SR. Myotube size was calculated by measuring myotube diameters in each condition. Representative images of siRNA, control siRNA and non-treated (SR) myotubes following 48 hours of SR are shown in Figure A.6A. Knockdown of MT1 and MT2 did not augment myotube atrophy as we had hypothesized. There was no difference in myotube diameter between any condition at 24 or 48 hours post-SR (Figure A.6 A and B). Similarly, under the control condition (high serum) MT1 and MT2 knockdown had no effect on myotube size or morphology (data not shown).

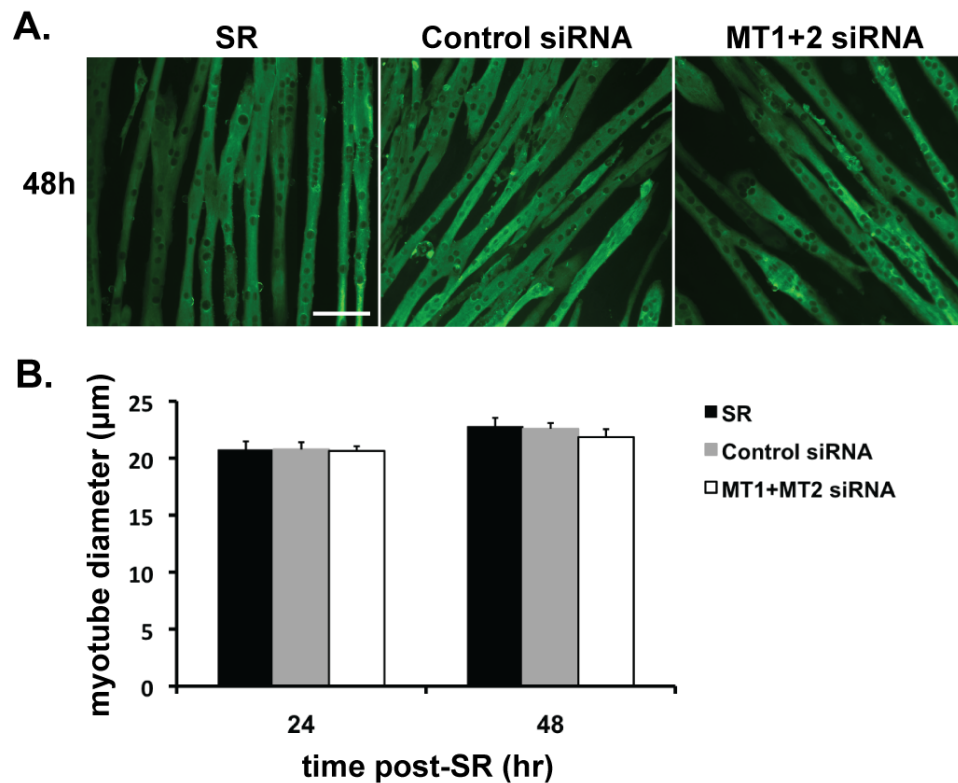


Figure A.6: MT1 and MT2 siRNA treatment does not affect myotube atrophy. A) Representative images of non-treated (SR), siRNA and control siRNA treated myotubes at 48 hours post-SR. Scale bar equals 100µm. B) Myotube diameter of non-treated (SR), siRNA and control siRNA treated myotubes at 24 and 48 hours post-SR. For myotube diameter, data are expressed in micrometers. Shown are means \pm SE from 3 independent experiments.

Discussion

As early as 1992, Kondo et al. (14) first observed changes in MT expression following an atrophy stimulus. Since then numerous reports in both animals and humans have shown increased MT gene expression in response to a muscle atrophy stimulus (30-32, 38, 45). Inquiries into the role that MTs play during atrophy, however, are recent. The prevailing hypothesis to explain rises in MT expression during atrophy has suggested that MTs may play a role in the oxidative stress response that accompanies muscle atrophy (49), thereby providing a source of protection from oxidative damage. This hypothesis is indeed plausible considering the reported antioxidant capacity of MTs in other tissues (46). Thus, we sought to test this hypothesis by knocking down the expression of the muscle-expressed MT isoforms MT1 and MT2 prior to applying a novel atrophy stimulus in cultured myotubes. Contrary to our hypothesis, we found that MT1 and MT2 knockdown did not affect myotube atrophy or oxidant activity.

The development of a novel in vitro model of muscle atrophy. Our experimental approach first necessitated that we develop an *in vitro* model of muscle atrophy in C₂C₁₂ myotubes. Several models of muscle atrophy have been developed previously (50, 51), most of which have relied on either the treatment of cell cultures with exogenous glucocorticoids or cytokines. However, previous work has noted the unpredictability of the effect of these agents on muscle culture response. For example, tumor necrosis factor-alpha (TNF- α) has been shown *in vitro* to reduce muscle protein content and promote muscle wasting (36). In contrast, a similar concentration of TNF- α has been demonstrated to have

mitogenic effects by promoting myoblast proliferation and inhibiting differentiation (50, 52). Likewise, in our hands, both glucocorticoid and TNF- α treatment has resulted in varying effects on myotube size (unpublished observations). Therefore, it became important for us to develop and characterize a more reliable model of muscle atrophy in cultured myotubes.

Here we have shown that a SR protocol is a simple and reliable stimulus to achieve atrophy of myotubes and loss of contractile protein content. Furthermore, we have demonstrated this effect by the simple manipulation of standard cell culture medium, rather than the treatment of cultures with exogenous agents. In this, we believe that we have developed a model of considerable value for the future study of muscle atrophy. Though others have experimented with serum stimulation as a means to produce myotube hypertrophy (53), we are the first to use the serum-stimulated (hypertrophied) myotubes as a platform to then induce a muscle atrophy response via subsequent serum reduction. Given that fetal bovine serum (serum used in the high serum growth medium condition) is known to be relatively growth factor rich and horse serum (serum used in SR condition) to be less so, we can speculate that the difference in growth factor concentrations of the two sera were responsible for driving the gains and losses of myotube size and protein. In support of this assertion, Yoshinouchi and Baserga (54) showed that fibroblasts cultured in medium containing a combination of IGF-1, PDGF and EGF in the absence of serum had a responded similarly to fibroblasts cultured in medium containing 10% serum. However, further experiments would be necessary to tease out the individual components of the serum responsible for the observed effect.

We then extended these analyses to examine changes in key signaling pathways that are associated with muscle atrophy *in vivo*. It has been shown that changes in AKT phosphorylation can regulate the induction of atrophy by control of the FOXO family of transcription factors, which control the expression of the ubiquitin E3 ligases atrogin1 and MuRF1 (13, 55). Thus, we measured both AKT phosphorylation and expression of the genes atrogin1 and MuRF1 in response to SR. We observed decreases in AKT phosphorylation, which correlated with concomitant increases in atrogin1 and MuRF1 mRNA abundance at 48 hours post-SR. However, it should be noted that myotube atrophy occurred primarily between 0 and 24 hours post-SR during which there were no measurable changes in AKT phosphorylation or atrogin1 and MuRF1 expression. This could mean that these molecular changes were not associated with myotube atrophy or that our detection methods were insufficiently sensitive to detect the initial changes in mRNA and protein abundance. While the bulk of the literature support a role for decreases in AKT phosphorylation following unloading or immobilization in rodents (4, 56, 57), others have reported muscle atrophy following unloading with no associated changes in AKT phosphorylation or its downstream targets (58). Furthermore, unloading or disuse-related atrophy can be driven by changes in signaling pathways distinct to the AKT signaling pathway (25, 59, 60).

The standard model for C₂C₁₂ cell differentiation (culture in GM followed by days of differentiation in DM) produces myotubes that are thinner than those generated in our paradigm (GM followed by DM followed by GM). This suggests that muscle fibers used

in other studies may not be as robust as those generated here. One implication of that possibility is that most studies with C₂C₁₂ cells may rely on myotubes that are partially starved, and thus do not reflect optimized cultures.

Muscle atrophy and oxidative stress following MT knockdown. We chose to test our hypothesis using siRNA directed at the two muscle-expressed MT isoforms. Immunoblot analysis confirmed that we were able to significantly reduce the amount of MT protein in our transfected cells. Because our antibody recognized multiple isoforms of MT, we also show via qRT-PCR specific reductions in our targets MT1 and MT2. Contrary to our hypothesis, targeted knockdown of the MT isoforms MT1 and MT2 did not affect myotube atrophy or alter the levels of intracellular cytosolic oxidant activity in our model. Our observations are consistent with recent data published by DeRuisseau *et al.* (61) who also failed to detect either a decline fiber cross-sectional area or endogenous antioxidant enzyme expression in soleus muscles following spinal cord transection in MT1^{-/-}/MT2^{-/-} mice. Interestingly, they did report greater soleus muscle contractile impairment following spinal cord transection in these null mice. These observations, together with our data, suggest that MTs do not directly influence the muscle atrophy response. DeRuisseau *et al.* (61) speculate that MTs are involved in the regulation of calcium handling during pathologic conditions and thus may result in diminished contractile force. These changes in calcium homeostasis may result from the metal binding capacity of MT proteins. For example, regulation of zinc and other metals is important in modulating muscle force and work capacity in skeletal muscle (62) and

likely is important in zinc finger transcription factor activity and thus overall gene expression under various stress stimuli

There are a number of published reports supporting of a role for MTs as protective antioxidants in various tissues (46, 63, 64). For example, Sun *et al.* (65) were able to demonstrate reduced cardiac myopathy in response to an oxidative stress stimulus using a MT-over-expressing transgenic mouse model. There is, however, little evidence supporting this role in skeletal muscle. Penkowa *et al.* (66) have documented increased expression of MT1 and MT2 protein in response to acute exercise, a stimulus known to increase oxidative stress. They suggested that the upregulation of MTs represented a response to counter oxidative stress-induced damage to muscle fibers, but there were no data provided to substantiate this suggestion. More recently, there was no difference shown in the expression of endogenous antioxidant enzymes in skeletal muscle of MT1 and MT2 deficient mice 7 days following spinal cord transection (61). Here we show, with the use of an *in vivo* intracellular probe, that SR results in an increase in oxidant activity (non-specific measure of oxidative stress), but that knockdown of MT1 and MT2 does not significantly alter that activity. Therefore, within the parameters of the experimental design, we could not support the hypothesis that MTs play a significant role in the endogenous antioxidant response to a muscle atrophy stimulus. Though clearly more work needs to be done to define a protective antioxidant role for MTs in skeletal muscle, the accumulating evidence would suggest that, if at all, they perform a minor function in this capacity.

In conclusion, we have described a novel, non-pathological *in vitro* model for both muscle atrophy and hypertrophy that may utilize more physiologically healthy muscle. The model requires only the manipulation of standard culture medium and thus may be of considerable value for future mechanistic studies of muscle atrophy. Using siRNA knock-down methodology, we were unable to demonstrate a role of MTs 1 and 2 in either atrophy or intracellular oxidant activity within the limitations of our experimental design. These findings are corroborated by recent knockout mouse data. Together, they lend strong support to the notion that, despite the reports of widespread mRNA increases during atrophy, MTs may not play a direct functional role in the atrophy response.

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