THE REQUIREMENT OF HSP70 IN THE REGULATION OF SKELETAL MUSCLE PLASTICITY: MUSCLE REGENERATION AND RECOVERY FOLLOWING INJURY

By

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To my parents, for their unwavering support, encouragement and love
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LIST OF ABBREVIATIONS

BrdU  bromodioxyuridine
CD68  cluster of differentiation 68
CSA   cross-sectional area
CTX   cardiotoxin
eMHC  embryonic myosin heavy chain
FoxO  forkhead boxO
H&E   hematoxylin and eosin
Hsps  heat shock proteins
IGF-1  insulin-like growth factor 1
IKK   inhibitory of κB kinase
iNOS  Inducible Nitric Oxide Synthase
MuRF1  muscle RING finger 1
NF-κB  nuclear factor κB
PBS   phosphate buffered saline
PCR   polymerase chain reaction
PI3K  phosphatidylinositol 3-kinase
TA    tibialis anterior
The inducible 70 kDa Heat shock protein (Hsp70) is expressed in virtually all cell types, and as a protein chaperone, plays a significant role in protecting against cellular damage and distress. Hsp70 protein levels are progressively decreased in skeletal muscle during aging, which is associated with the age-related decline in skeletal muscle mass, function and regenerative potential. Importantly, Hsp70 is also decreased during skeletal muscle disuse, and restoration of its levels is sufficient to prevent muscle fiber atrophy. Furthermore, Hsp70 overexpression has also been shown to promote both muscle regeneration and functional recovery following injury, further demonstrating the breadth of cellular protection provided by Hsp70 to skeletal muscle. While the protective nature of Hsp70 demonstrated in these studies may indeed be related to its ubiquitous role as a general protein chaperone, we hypothesize that Hsp70 is in fact an indispensable protein in skeletal muscle necessary for normal skeletal muscle plasticity. In this regard, we hypothesize that a decrease in Hsp70 protein levels alone, as occurs during both muscle disuse and during the aging process, is sufficient to both alter skeletal muscle homeostasis under normal conditions and impede the normal
regenerative and recovery process that occurs following injury and modified muscle use. The current study tested these hypotheses through the use of genetically modified mice which lack Hsp70 (Hsp70<sup>−/−</sup> mice). We found that muscles from Hsp70<sup>−/−</sup> mice had significantly smaller skeletal muscle fibers than those of WT mice under normal physiological conditions, and were severely impaired in their ability to recover following injury due to cardiotoxin or modified muscle use. Specifically, Hsp70<sup>−/−</sup> mice displayed increased numbers of necrotic muscle fibers, increased mRNA levels of pro-inflammatory cytokines and calcium-activated proteases, increased numbers of CD68+ macrophages and deficits in regenerating fiber size that persisted up to 6-weeks post-injury. Interestingly, injured muscles from Hsp70<sup>−/−</sup> mice also developed calcifications and significant fibrosis, characteristic of dystrophic muscle phenotypes. Collectively, findings from this study demonstrate that Hsp70 is necessary for normal skeletal muscle fiber size, and is further required for successful muscle regeneration and recovery following muscle injury.
CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Heat shock protein 70 (Hsp70) expression is induced in response to a variety of physiological insults (55, 66), and is believed to be a significant player in protecting skeletal muscle from cellular damage and distress. The mechanistic nature of Hsp70-mediated cellular protection is believed to be related to both its role as a protein chaperone in regulating protein turnover (5, 27) and its ability to modulate cell signaling pathways. As a chaperone, Hsp70 plays a dual role in preventing global protein denaturation and degradation following mild to moderate protein damage (5, 66), while promoting the degradation & clearance of irreversibly damaged proteins. In terms of its role in regulating cell-signaling events, Hsp70 overexpression studies have demonstrated that Hsp70 can regulate the activities of various signaling proteins and transcription factors, including jun N-terminal kinase (JNK) (10), p38 (20), nuclear factor κB (NF-κB) (10, 20, 71, 80) and forkhead boxO (FoxO) (79, 80). Importantly, in skeletal muscle, these signaling proteins are involved in the regulation of various muscle remodeling processes, including myogenesis and muscle growth (3), regeneration following injury (58) and muscle atrophy (6, 33, 35, 95). Perhaps more importantly in these studies, Hsp70 overexpression ultimately protected against disruptions in muscle homeostasis in response to varying physiological and pathophysiological stimuli. Protection against muscle atrophy (80) and muscle damage (4), and promotion of regenerative processes were all achieved through muscle-specific Hsp70 overexpression studies, highlighting a seemingly critical role for Hsp70 in regulating skeletal muscle plasticity. Interestingly, Hsp70 is down-regulated during the aging
process, in which reductions in muscle mass and function and impaired regeneration following injury are all a significant problem. However, it is currently unknown whether a decrease in Hsp70 alone can cause disruptions in these cellular processes which ultimately regulate skeletal muscle mass and function. Determining the relative phenotype of skeletal muscles which lack Hsp70, and further determining whether these muscles can successfully regenerate and recover following injury would provide critical information not currently known on the biological requirement of Hsp70 in skeletal muscle remodeling. Importantly, knowledge gained from these studies may extend the clinical potential for Hsp70 in the treatment of a variety of skeletal muscle disorders, and would ideally enhance the momentum toward the development of Hsp70-targeted therapeutics that can rapidly translate to future clinical studies.

**Heat Shock Protein 70**

**Overview**

Heat shock proteins (Hsps) are a group of proteins which act as cellular chaperones, assisting in the folding of nascent polypeptides and the re-folding of damaged proteins. Hsps are generally separated into two groups based on their molecular weight, and includes the high-molecular weight Hsps and the low-molecular weight Hsps. The Hsp70 family belongs to the ATP-dependent, high-molecular-weight group of Hsps (87), along with the Hsp90 and Hsp60 families. Of the cytoplasmic Hsp70 family members the two most studied members are Hsc70, which is constitutively expressed, and the stress-inducible Hsp70. As cellular chaperones, Hsc70/Hsp70 function to help fold nascent proteins and re-fold damaged proteins into their correct tertiary structures (16). Importantly however, when proteins are irreversibly damaged or even designated for protein degradation, Hsc70/Hsp70 can help shuttle these proteins
to the proteasome for their subsequent degradation (39). This involvement of Hsc70/Hsp70 in protein degradation plays an important role in preventing protein aggregation and promoting cellular homeostasis.

The relative co-chaperones which interact with Hsc70/Hsp70 appear to play important roles in the fate of Hsc70/Hsp70 client proteins. The co-chaperones CHIP (c-terminus of Hsp70/Hsc70 interacting protein) and BAG-1 assist in Hsc70/Hsp70-mediated degradation of client proteins (11, 59, 88). CHIP is a ubiquitin ligase that when in complex with Hsc70/Hsp70, can ubiquitinate client proteins allowing for their subsequent degradation through the ubiquitin-dependent proteasome system (59). BAG-1 interacts with the 20S core and 19S subunit of the proteasome, and facilitates the release of Hsp70 client substrates to the proteasome (45). In contrast, the co-chaperones Hip and Hop assist Hsp70 in the folding and re-folding of protein substrates (18, 30). Therefore, dictated by which co-chaperone proteins Hsc70/Hsp70 interacts with, Hsc70/Hsp70 can either promote protein folding and stability, or promote protein degradation. Since Hsc70 and Hsp70 are believed to be functionally similar due to their high homology, insight into the relative importance of these separate gene products can be gained from the conditions in which they are differentially regulated. In this body of work the specific focus will be on the role of the stress-inducible Hsp70 in skeletal muscle. Hsp70 is altered in skeletal muscle in response to numerous physiological and environmental stimuli, which suggests a role for this protein in the subsequent skeletal muscle remodeling processes which simultaneously occur in response to these stimuli.

**Hsp70-mediated Cellular Protection**

Various environmental stimuli, including free-radicals, oxygen or nutrient deprivation and mechanical injury may result in the loss of protein and cellular integrity
resulting in alterations in cellular homeostasis (21). Elevation of Hsp70 during these stress conditions promotes cell survival and helps return cells to their normal homeostatic state (44, 63), which is critical to maintaining cellular function. The protective nature of Hsp70 has been linked to both its general chaperone function, as well its ability to regulate various kinases involved in cellular signaling, including the stress kinases JNK and p38 (20), and the Inhibitor of κB kinase (IKK) complex (71). Regardless of the precise mechanism of protection, it can be concluded from these studies that induction of Hsp70 is a highly protective mechanism involved in cellular stress tolerance.

However, as cells age and are exposed to continuous cycles of cellular stress, a loss of cellular integrity is sometimes unavoidable. Fortunately, many cell types undergo numerous cycles of proliferation throughout their lifetime, such that older and/or irreversibly damaged cells that have severely declined in their cellular integrity can undergo programmed cell death, leaving behind sufficient levels of healthier or “newer” cells to carry out tissue function. Some tissues, like the skin, replace cells every few weeks or months. In contrast, some tissues are comprised of cells that are post-mitotic, such as neuronal cells in the brain and skeletal muscle fibers (cells) in skeletal muscle. These post-mitotic cells do not undergo further cellular proliferation, and may live for a lifetime. Therefore, maintenance of protein and cellular integrity in post-mitotic cells is especially critical for cellular and tissue function. The importance of Hsp70 in promoting cellular integrity in post-mitotic neuronal cells, especially in aged populations, has been shown in numerous studies. Hoshino et al recently demonstrated the preventative effect of neuronal-specific Hsp70 transgenic expression in a rodent model of Alzheimer’s
disease (AD) (31). Indeed, the build-up and aggregation of beta-amyloid protein, which is involved in the pathogenesis of AD, was prevented by Hsp70 overexpression, as were the behavioral manifestations of AD, such as memory loss (31). Hsp70 overexpression also interferes with the pathology of Amyotrophic Lateral Sclerosis (22) and Parkinson's disease (99), additional diseases whose pathology is characterized by defects in protein quality control that typically begin to occur later in life.

As previously mentioned, skeletal muscle fibers are also post-mitotic cells. Although multi-nucleated skeletal muscle fibers do undergo turnover of cellular nuclei through apoptosis of existing fiber nuclei and replacement of these nuclei through the fusion of activated muscle satellite cells (7), individual muscle fibers may remain for a lifetime. Therefore, similar to neuronal cells in the maintenance of brain and motor neuron function, preserving protein integrity and cellular homeostasis in skeletal muscle fibers is especially critical to the maintenance of skeletal muscle function. Due to the roles of Hsp70 in regulating cellular homeostasis, Hsp70 has for many years been speculated to play an important role in skeletal muscle to promote homeostasis and overall muscle health (42). In this regard, Hsp70 protein expression is induced in skeletal muscle in response to exercise and muscle activity, which is believed to protect the muscle from alterations in protein and structural integrity that may occur during these conditions due to elevations in muscle temperature, mechanical strain, oxidative stress and changes in pH, to name just a few. Importantly, Hsp70 protein expression declines with age, and the relative ability to increase Hsp70 in skeletal muscle in response to exercise and muscle activity, is also blunted with age (51, 96). This decrease in Hsp70 is associated with the age-related reductions in skeletal muscle...
mass, function and regenerative potential, as increasing the levels of Hsp70 in aged mice through transgenic expression results in increased function under normal conditions and enhanced recovery following exercise-induced muscle damage when compared to aged wildtype mice (50). This evidence which shows that Hsp70 overexpression can protect against these age-related deficits in skeletal muscle plasticity further supports the importance of Hsp70 in regulating cellular homeostasis and function. Therefore, the speculation that decreased levels of Hsp70 alone may contribute to these age-related deficits in skeletal muscle is not without warrant (51).

Prior to discussing further the evidence which supports Hsp70 in regulating skeletal muscle plasticity, the next chapter will provide an introduction to skeletal muscle and its extraordinary abilities to adapt to its environment and recover following even the most severe cases of cellular stress and damage. Based on the functional properties of Hsp70 discussed above, hopefully one will appreciate the potential roles that Hsp70 may specifically play in regulating the skeletal muscle processes that provide muscle its plastic nature.

**Skeletal Muscle Plasticity**

In biology, plasticity can be defined as the ability to adapt to environmental and/or functional demands, which may be reflected by changes in both structural and/or functional phenotype. In skeletal muscle, plasticity may be reflected by changes in fiber cross-sectional area and length as well as fiber-type shifts, which may occur following modified muscle activity (76). However, perhaps the most extraordinary plastic feature of skeletal muscle is its ability to self-renew. This ability to self-renew or regenerate is provided by skeletal muscle satellite cells. Satellite cells are specialized muscle stem cells that are located adjacent to muscle fibers, underneath the basement membrane.
Activation and fusion of satellite cells into muscle fibers allow for post-natal muscle growth and maturation, as well as muscle re-growth, repair and even complete regeneration following injury (7). Interruptions in any of these skeletal muscle processes can result in significant impairments in skeletal muscle function. Indeed, numerous skeletal muscle disorders, including the genetic muscular dystrophies (97) and inflammatory myopathies (48) are characterized by significant impairments in skeletal muscle function due to interruptions in these critical skeletal muscle processes. Even various physiological conditions such as prolonged muscle inactivity and aging (47), and pathophysiological conditions such as cancer and sepsis (56), can result in impaired muscle function due to the downstream consequences of these conditions on these cellular processes in skeletal muscle. Importantly, these deficits in skeletal muscle function often have devastating consequences to patient health and even survival (56, 97). Therefore, understanding the detailed mechanisms underlying skeletal muscle plasticity is the foundation for future clinical treatments which aim to exploit the plastic nature of skeletal muscle to treat, and ideally cure these devastating conditions. As the current work will focus on the importance of Hsp70 in skeletal muscle plasticity, a more detailed introduction to the skeletal muscle processes that give muscle its plastic nature will be discussed, focusing specifically on the regulation of skeletal muscle mass (atrophy and hypertrophy) as well as muscle re-growth and regeneration following injury.

**Regulation of Skeletal Muscle Mass**

The maintenance of skeletal muscle mass occurs through balancing protein degradation and protein synthesis, which is mediated through both circulating systemic and local growth and growth-inhibitory factors (e.g. Insulin-like Growth Factor 1 [IGF-1])
and myostatin) and the mechanical load placed on the muscle (23, 34, 74). In response to increased skeletal muscle loading or administration of growth factors, muscle fibers can hypertrophy or increase in mass and fiber cross sectional area (24). On the other hand, skeletal muscle can decrease in size (atrophy) in response to decreased muscle loading, in response to certain circulating factors such as inflammatory cytokines that are elevated during various disease conditions (2), or in response to deficits in normal growth signaling pathways (34).

Skeletal muscle atrophy occurs in response to a variety of both physiological and pathophysiological stimuli, and contributes to profound losses of muscle mass and whole body strength (34). Among the physiological stimuli which trigger the loss of muscle mass is muscle disuse, prolonged muscle inactivity that results in reduced mechanical loading or tension on the muscle (26, 90). Atrophy resulting from cast-immobilization of a limb, bed rest, or a general inactive lifestyle each fall under the category of disuse muscle atrophy, as does atrophy resulting from interruptions in neural input to the muscle, such that occurs during functional denervation in elderly patients (46). In contrast, cachectic muscle wasting is a form of pathophysiological wasting that often accompanies chronic diseases such as cancer (2) and chronic heart failure, and more acute conditions such as sepsis (28). Although patients affected by these conditions may suffer from some level of disuse wasting as a consequence of inactivity due to their disease, cachectic wasting (cachexia) is believed to predominately result from increased levels of systemic circulating factors such as inflammatory cytokines that are elevated by the host immune system in response to the disease (93). In addition to immune-derived factors, tumor-secreted factors in cancer patients, and
blood-borne lipopolysaccharide from the bacterial infection in septic patients, are also implicated in the pathogenesis of muscle wasting (93). Therefore, although there are multiple distinct triggers of muscle wasting, in the clinical population, muscle wasting is often a complex condition that may involve numerous underlying triggers. Importantly, the loss of muscle mass not only affects muscle strength and function, it also increases the risk for metabolic disorders such as diabetes, contributes to prolonged and/or impaired recovery following hospital stays, and in the most severe cases, contributes to increased mortality (56, 94). Therefore, improving our understanding of how skeletal muscle atrophy is regulated is clinically significant for not only combating or alleviating the muscle atrophy itself, but enhancing patient health and survival.

Despite the numerous upstream triggers of skeletal muscle atrophy, the loss of skeletal muscle mass and function results from the preferential loss of skeletal muscle contractile proteins (19, 83). During normal physiological conditions, skeletal muscle contractile proteins are degraded at a similar rate in which they are synthesized, which results in an overall maintenance of healthy muscle proteins and maintenance of muscle mass. In response to atrophic stimuli, protein degradation through the ubiquitin proteasome system is elevated significantly (89), while protein synthesis coordinately declines, resulting in a net decrease in skeletal muscle contractile protein. Since these changes in protein turnover ultimately dictate whether a muscle will atrophy, hypertrophy or remain a certain fiber size, understanding how these cellular processes are regulated in skeletal muscle has become a major research focus in recent years.

Numerous proteins and signaling pathways have been identified to regulate skeletal mass through modulating the balance between protein synthesis and protein
degradation (24). Growth signaling through Akt both stimulates protein synthesis through activating mTOR signaling and inhibits protein degradation pathways through phosphorylating and inhibiting the Forkhead boxO (FoxO) transcription factors (75, 85). FoxO transcription factors are activated during atrophic conditions including muscle disuse (75, 80), cancer and sepsis (82), and their activation is required for normal muscle wasting (72, 79). FoxO transcription factors cause muscle atrophy via their transcriptional regulation of multiple genes involved in proteolysis, including genes involved in the ubiquitin proteasome system (75). FoxO also regulates genes involved in autophagy (49), which is an important cellular process involved in the basal turnover of cellular organelles during normal physiological conditions that is accelerated during certain conditions of muscle atrophy (49). Importantly, our lab group recently demonstrated that decreased levels of FoxO signaling under normal physiological conditions can lead to skeletal muscle hypertrophy (72). Although this is likely related in part to decreased levels of basal protein turnover, this hypertrophy also required protein synthesis and involved both satellite-cell proliferation and fusion, and decreased myostatin signaling. Therefore, it appears that FoxO regulates not only proteolytic pathways during atrophic conditions, but also plays an important role in preventing muscle fiber hypertrophy during normal physiological conditions.

There is also strong evidence that NF-κB signaling is involved in the regulation of skeletal muscle mass (68). Indeed NF-κB transcriptional activity is increased during atrophic conditions (6, 33, 67), and inhibition of NF-κB via genetic manipulation of various proteins in the NF-κB signaling pathway, including p50, Bcl3, IκBα, IKKα and IKKβ are all sufficient to inhibit the normal atrophy program (6, 32, 35, 58, 95). While the
precise role in which NF-κB activation contributes to muscle loss during atrophy conditions is still not well defined, the atrophy gene MuRF1 is a known NF-κB target gene (6). While numerous other signaling pathways and proteins have also been identified to regulate skeletal muscle mass during normal physiological and pathophysiological conditions (74), due to the scope of the current study, these signaling pathways will not be discussed.

**Muscle Regeneration and Recovery following Muscle Injury**

Skeletal muscle not only has the ability to change the size of individual muscle fibers (atrophy/hypertrophy), it also has a remarkable capacity to repair and even replace damaged muscle fibers following injury (7). This capability of skeletal muscle to “re-grow” muscle fibers is termed muscle regeneration, and involves a specialized muscle stem cell known as a satellite cell. Following muscle injury (mechanical or chemical), factors released by the injured muscle and by resident and invading inflammatory cells stimulate these normally quiescent satellite cells to activate, proliferate, and eventually differentiate and fuse with either existing muscle fibers or to other myogenic cells to generate new myotubes (7, 91). Impairments in any one of these coordinated events which lead to muscle regeneration profoundly limit the ability of skeletal muscle to recover following muscle injury (7, 91).

Muscle injury may occur in response to a variety of physiological insults including crush injury, but more commonly, following mechanical strain on the muscle due to eccentric or damaging muscle contractions, and during muscle reloading following prolonged periods of muscle unloading and disuse (7, 47). Although mechanical injury is the primary physiological cause of muscle injury, chemicals (reactive oxygen species,
reactive metals, and/or toxins) can also induce muscle injury. Regardless of the nature of acute muscle damage, the general time course in which specific myeloid cell populations accumulate in the muscle and myogenesis proceeds is similar, and well-defined (7, 91). Indeed, following the initial bout of injury the innate immune response is activated, leading to the infiltration of neutrophils, which may peak in concentration between 6 and 24 hours post injury (7, 91). Neutrophils play an important role in the release of myeloperoxidase, which induces muscle membrane damage and amplification of muscle injury (60, 62). Importantly, soon after the invasion of neutrophils, phagocytic pro-inflammatory (M1) macrophages begin to invade and peak in concentration 24-48 hours post injury, followed by a sharp decline in numbers thereafter (91). These pro-inflammatory macrophages help remove necrotic and damaged muscle fibers and debris, and also release both growth factors and pro-inflammatory cytokines that are involved in stimulating satellite cell activation and proliferation (86, 91). However, overproduction and/or sustained release of cytokines by these macrophages can also inhibit regenerative processes. Therefore, soon after the peak in the pro-inflammatory M1 macrophages, there is shift in these macrophages toward an anti-inflammatory (M2) phenotype (91). This shift in macrophage phenotype towards an anti-inflammatory phenotype corresponds with the shift from the proliferative phase of muscle regeneration to the differentiation phase. Indeed, these M2 macrophages secrete anti-inflammatory cytokines that are involved in deactivating the pro-inflammatory immune response and promoting muscle differentiation, repair and growth of regenerating fibers (91, 92). Depending on the severity of the damaging stimuli, restoration of normal fiber architecture usually occurs around 10 days following
muscle injury, followed by restoration of mature fiber cross-sectional area by 3-4 weeks post-injury (7).

**Hsp70 Overexpression and Skeletal Muscle Plasticity**

Hsp70 has long been speculated to play a role in protecting skeletal muscle under stressful stimuli, as discussed earlier. Hsp70 is elevated in skeletal muscle following exercise (29, 52, 57), as well as during periods of muscle re-growth following muscle disuse (78). In contrast, Hsp70 is decreased during periods of muscle inactivity (9, 40, 80) when muscles undergo remodeling to reduce their size according to the new reduced mechanical load on the muscle. Given this association of elevated Hsp70 levels during periods of muscle activity, growth and regeneration, and decreased levels during periods of muscle inactivity and muscle atrophy, the idea that Hsp70 may positively regulate muscle mass is not without warrant. In support of this notion, previous studies using heat stress to increase heat shock proteins in skeletal muscle have shown that heat stress can provide some level of protection against disuse muscle atrophy (77) and can promote muscle regrowth (78). Similarly, heat stress prior to or following muscle injury due to bupivacaine (64) or cardiotoxin (36) promoted satellite cell activation and earlier recovery of regenerating skeletal muscle fiber size, further indicating a role for heat shock proteins in regulating muscle regeneration. However, as heating may alter the activation of multiple cellular signaling pathways and differentially regulate the protein expression of many proteins in addition to Hsp70, these heating studies are only suggestive evidence of the role that Hsps and Hsp70 may play in regulating skeletal muscle plasticity.

More direct evidence to specifically implicate Hsp70 in the regulation of skeletal mass and function comes from studies which have genetically overexpressed Hsp70.
Muscles from skeletal muscle-specific Hsp70 overexpressor mice recover quicker, both morphologically and functionally, following muscle damage due to eccentric lengthening contractions and are also protected from the age-related loss in specific muscle force (50). Similarly, muscles from Hsp70 transgenics show less damage following cryolesioning injury compared to WT, as indicated by less inflammatory cell infiltration and numbers of necrotic fibers one day post-injury (53). Muscles from Hsp70 transgenics in this study also showed lower levels of satellite cell activation and proliferation. This finding was speculated to be related to the decreased muscle damage in the Hsp70 transgenics, which would thereby result in a decreased need for satellite cell-mediated repair of muscle fibers. An additional study using plasmid-mediated overexpression of Hsp70 following freeze injury further demonstrated that Hsp70 enhances regenerating muscle fiber size (54). Together these studies provide strong evidence that Hsp70 indeed is sufficient to both protect skeletal muscle from damage in the event of injury, and promote muscle regeneration and recovery. Importantly, Hsp70 protein levels are increased in muscle biopsies from patients diagnosed with However, whether Hsp70 is required for muscle regeneration and recovery in the event of muscle injury is not currently known.

In addition to a role for Hsp70 in muscle recovery following injury, Hsp70 has also been shown to protect against skeletal muscle atrophy during muscle disuse (80). Indeed, Hsp70 levels are significantly downregulated during muscle disuse, and plasmid-mediated restoration of Hsp70 in skeletal muscle prevents greater than 75% of the fiber atrophy (80). Findings from this study indicate that a decrease in endogenous Hsp70 during disuse may be an important event allowing the atrophy program to
proceed. Since Hsp70 protein is also decreased in aged skeletal muscle (14, 51) it may also be speculated that the age-related loss of muscle mass may similarly be related to the decreased levels of Hsp70. However, whether a decrease in Hsp70 protein alone is sufficient to decrease skeletal muscle fiber size has not been established.

Important additional information gained from experiments performed in these studies in skeletal muscle and in studies from other cell types, indicate that Hsp70 can modulate specific intracellular signaling pathways. Indeed, Hsp70 overexpression represses NFκB signaling in skeletal muscle (80) a pathway whose activation is well established to be necessary for muscle fiber atrophy during various atrophic conditions (6, 32, 35, 95). Signaling through NF-κB also limits skeletal muscle growth (3, 68) and impairs muscle regeneration (58). Additionally, Hsp70 overexpression also represses FoxO signaling in skeletal muscle (79, 80), another pathway known to be required for muscle fiber atrophy (72, 75, 79) and which also limits skeletal muscle fiber hypertrophy under normal conditions (72, 73). Hsp70 also decreases the activity of JNK in skeletal muscle, a stress-kinase whose activity in skeletal muscle is linked to inflammatory signaling and insulin resistance (10). Indeed, muscle specific Hsp70 transgenics are protected from diet- and obesity-induced glucose intolerance and insulin resistance, which was tightly associated with repression of JNK and IKK (10). Therefore, the ability of Hsp70 to regulate these critical cell signaling proteins in skeletal muscle may explain, in part, the findings that up-regulation of Hsp70 can preserve muscle mass under catabolic conditions, and enhance muscle recovery following injury. However, no studies currently exist to demonstrate the physiological requirement of Hsp70 for any skeletal muscle adaptation. Therefore it is currently unknown whether Hsp70 is indeed
indispensable for normal skeletal muscle mass and phenotype during normal conditions and whether Hsp70 is further required for the coordinated cellular events that occur in skeletal muscle following injury that allow for normal muscle re-growth and recovery.

**Hypotheses and Aims of the Current study**

Numerous studies from independent lab groups have provided clear evidence to suggest a critical role for Hsp70 in regulating skeletal muscle plasticity. To summarize, these studies show that, 1) skeletal muscle expression of Hsp70 is decreased progressively with age, and is associated with the age-related loss of muscle mass and regenerative potential; 2) Hsp70 is decreased during periods of muscle disuse, and restoration of its levels can prevent fiber atrophy; 3) Hsp70 overexpression enhances regenerating fiber size and functional recovery following muscle injury and; 4) Hsp70 represses the activation of multiple signaling pathways in skeletal muscle which negatively regulate skeletal muscle mass and regenerative potential. Despite this evidence and the wide speculation that Hsp70 is central to the ability of skeletal muscle to withstand and respond to stress, there are currently no studies that have directly considered the requirement of Hsp70 in any type of skeletal muscle adaptation. Therefore, the aim of the current study was to test the central hypothesis that skeletal muscle expression of Hsp70 is *indispensable* for normal skeletal muscle phenotype under normal conditions, and is further necessary for the normal recovery process following muscle injury.

The current study tested the above hypothesis through the use of wild-type and genetically modified mice that lack Hsp70 (Hsp70−/− mice). Skeletal muscle fiber-type distributions, fiber cross-sectional area, total fiber numbers and general morphology were assessed in wild-type (WT) and Hsp70−/− mice under normal physiological
conditions, to determine whether Hsp70 is necessary for normal muscle phenotype. To further determine whether Hsp70 is necessary for normal muscle regeneration and recovery following muscle injury we utilized two distinct models that are well used in the study of muscle injury and recovery, cardiotoxin injury and modified muscle use. Both biochemical and histological measurements were used at various time points post-injury to assess the relative levels of muscle damage, inflammation and regenerative capabilities of muscles from WT and Hsp70−/− mice. Lastly, rescue experiments were performed via the electroporation of Hsp70-EGFP or EGFP specifically in skeletal muscles of Hsp70−/− mice either 4 days prior to or 4 days following muscle injury, to determine whether any deficits in skeletal muscle regeneration and recovery were indeed due to the lack of muscle derived Hsp70 at these time points.
CHAPTER 2
MATERIALS AND METHODS

Animals

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. B6129S7-Hspa1a/Hspa1b<sup>tm</sup> mice (hereafter referred to as Hsp70<sup>−/−</sup> mice) were originally generated at the US Environmental Protection Agency (13) and were purchased from the Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis. Their wild-type counterparts, B6129SF2/J mice (hereafter referred to as WT mice) were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in a sterile, pathogen-free, temperature-controlled facility on a normal 12-hr light/dark cycle, and standard diet and water were provided ad libitum.

Cardiotoxin Model of Muscle Injury

To induce muscle degeneration/regeneration, 100 µl of 10 µM cardiotoxin (Calbiochem) dissolved in PBS was injected into either the right or left tibialis anterior (TA) muscle of 6-8 week old WT or Hsp70<sup>−/−</sup> mice. Cardiotoxin is a protein kinase C-specific inhibitor, and its injection into skeletal muscle induces muscle depolarization, contraction and subsequent fiber necrosis in greater than 90% of muscle fibers (100). Animals were sacrificed with a lethal dose of pentobarbital and muscles were harvested 1, 4, 6, 16, 28 or 42 days post-cardiotoxin injection. The uninjured, contralateral TA muscles from each animal served as controls.

BrdU Labeling of Proliferating Cells

To measure cellular proliferation in TA muscles in response to cardiotoxin-injury, mice were injected intraperitoneal (IP) daily for 6 days with 50mg/kg Bromodeoxyuridine
(BrdU) dissolved in sterile saline beginning 6 hours prior to cardiotoxin-injury. BrdU is a thymidine analogue that is incorporated into DNA during DNA replication, which occurs during cellular proliferation. Since skeletal muscle satellite cells proliferate extensively following muscle injury due to cardiotoxin, staining for BrdU intensity and localization in skeletal muscle fibers in muscle cross-sections can provide important information on satellite cell proliferative capacity and regeneration involving satellite cell activation. Injured and contralateral non-injured TA muscles were surgically removed 6 days post-cardiotoxin-injury, embedded in OCT freezing medium and frozen in liquid nitrogen-cooled isopentane prior to storage at -80°C.

**Hind Limb Immobilization/Reloading Model of Muscle Injury**

Muscle reloading-injury was induced via bilateral hind limb cast-immobilization with ankles in the plantar-flexed position for 10 days as described previously (ref), followed by cast-removal and reambulation for either 3 or 10 days. Briefly, a layer of protective padding was applied to both hind limbs and the lower abdominal area (Medipore). Extra-fast-drying plaster (3M) was then wetted and applied over the protective padding and allowed to dry. A thin layer of casting tape (3M) was then applied over the plaster to prevent mice from chewing through the plaster casts. Skeletal muscle unloading/reloading-injury in the soleus has been demonstrated by numerous lab groups following both hind limb immobilization as performed in the current study (17) as well as following hind limb suspension (61, 91).

**Histology**

Skeletal muscles from Hsp70−/− or WT mice to be used for histology were removed, embedded in OTC and immediately frozen in isopentane cooled in liquid nitrogen prior to storage at -80°C. Prior to sectioning, muscles were equilibrated at -20°C for 1 hour. A
microtome cryostat was then used to cut 10-μm-thick serial transverse sections, which were transferred to positively charged glass slides. Sections were allowed to dry for 1 hour at room temperature prior to freezing at -80°C until further processing or stained immediately using H&E to determine morphology. All stained sections were visualized using a Leica DM5000B microscope (Leica Microsystems Bannockburn, IL). Images of entire muscle cross-sections were captured for 4 sections separated by at least 50 μM per muscle. Each histological analysis was performed on at least 4 muscles per group.

**Hematoxylin & Eosin (H&E) Staining**

H&E staining of muscle cross-sections was performed as described previously (17). Briefly, slides were brought to room temperature prior to sequential submersions in the following solutions: 100% ethanol for 1 min, 70% ethanol for 1 min, dH₂O for 2 min and Gill’s Hematoxylin for 2 min. Sections were then washed thoroughly in dH₂O followed by sequential submersions in the following solutions: Scott’s Solution for 15 seconds, dH₂O for 2 seconds, 70% ethanol for 1 minute, Eosin for 2 minutes, 95% ethanol with gentle shaking for 1 minute, 100% ethanol for 30 seconds and Xylene for 2-3 minutes. Slides were allowed to dry for 30 minutes and then mounted with glass cover-slips using Permount.

**Von Kossa and Trichrome Staining**

Slides containing fresh frozen muscle cross-sections (10 μm) were sent to the University of Florida Pathology Core Facility for trichrome staining to detect collagen (stains blue) or Von Kossa staining to detect calcium deposits (stains black).
**Immunohistochemistry**

For immunohistochemistry experiments, sections were permeabilized for 5 minutes in 0.1% Triton-X-100, washed 2 X 5 minutes in PBS and blocked in Pierce Superblock for 15 minutes. Sections were then incubated in primary antibody diluted in blocking buffer at 4°C overnight in a humid chamber. Primary antibodies for the following proteins were used: embryonic myosin heavy chain (1:100, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and laminin (1:275, Sigma Aldrich). Sections were washed 3 X 5 minutes in PBS prior to incubation with the appropriate fluorescently-conjugated secondary antibodies from Invitrogen in blocking solution for 1 hour at room temperature. CD68-AlexaFluor 488-conjugated primary antibody (1:175, AbD Serotec), when used, was added during the secondary step. Sections were then washed 3 X 5 minutes in PBS and mounted with cover-slips using VECTASHIELD® fluorescence mounting medium with or without Dapi (Vector Laboratories, Inc).

**BrdU Immunostaining**

Muscle cross-sections were rehydrated briefly in PBS prior to antigen retrieval with citrate buffer (1.8mM citric acid and 8.2 mM sodium citrate, pH 6.0) as described previously (72). Endogenous peroxide activity was inhibited via incubating sections in 0.3% hydrogen peroxide for 5 minutes. Sections were washed in PBS and then incubated in primary antibody (anti-BrdU, 1:100, Roche Diagnostics, Indianapolis, IN, USA, and anti-laminin, 1:275, Sigma Aldrich) overnight at 4°C. The following day sections were washed extensively with PBS prior to incubation with secondary antibodies (Alexa Fluor 594 anti-mouse IgG and Alexa Fluor 488 anti-rabbit IgG; Invitrogen) for 1 hour at room temperature. Sections were washed extensively, and then
mounted with cover slips using VECTASHIELD® fluorescence mounting medium containing Dapi (Vector Laboratories).

**Quantification of Muscle Necrosis, Muscle Regeneration and Fiber CSA**

Quantitative morphological analysis of TA and soleus muscle cross sections stained with H&E was performed on 4 serial cross-sections (separated by at least 50 µM per muscle. Representative images from non-overlapping fields were visualized and captured for each section using a Leica DM5000B microscope (Leica Microsystems Bannockburn, IL). Fibers undergoing regeneration were identified as those containing centrally located nuclei. Regenerating fiber cross-sectional area (CSA) and the number of regenerating fibers in entire cross-sections was calculated in muscles 16 days post-cardiototoxin injection or following 10 days of muscle reloading, using Leica Application Suite software (version 3.5.0). This software was similarly used to calculate the average CSA of fibers in serial sections from muscles 28 and 42 days post-cardiototoxin, except that the CSA of all fibers in entire cross-sections were measured.

Muscle fibers in early-stage necrosis were identified on H&E stain as fibers with reduced eosinophilic staining and clear fiber borders, with inflammatory cells in or surrounding the muscle fiber. Muscle necrosis was quantified via counting and averaging the total number of necrotic muscle fibers per field in entire muscle cross-sections in at least 4 serial sections separated by at least 50 µm per muscle, n=4 muscles per group.

**RNA Isolation, cDNA Synthesis and qRT-PCR**

Muscles to be used for RNA isolation were immediately removed, snap-frozen in liquid nitrogen and stored at -80°C. Muscles were quickly minced in TRIZol reagent,
1:10 (wt/volume), followed by thorough homogenization using a Polytron homogenizer and stored at -80°C until further processing. Prior to RNA isolation, muscle homogenates were thawed to room temperature, vortexed thoroughly, and RNA isolated using a chloroform-based method according to the TRIzol manufacturer’s directions as described previously (80). RNA concentrations ($A_{260}$) and quality ($A_{260}/A_{280}$) were assessed using a spectrophotometer. Prior to qRT-PCR, RNA (1ug) was reverse transcribed using Ambion’s RETROscript first strand synthesis kit (Ambion, Austin, TX, USA). Resulting cDNA was diluted 1/60 in dH$_2$O and used as a template for real-time PCR using universal primers purchased from Applied Biosystems. A 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to detect PCR products and quantification was performed using a relative standard curve.

**RT$^2$ Profiler PCR Arrays**

RNA isolated as described above was reverse transcribed to cDNA using an RT$^2$ First Strand Kit following the manufacturer’s instructions using 1μg RNA as starting template (SA Biosciences). RT$^2$ SYBR Green/ROX qPCR Master Mix and the Mouse Skeletal Muscle: Myogenesis & Myopathy PCR Arrays (96-well Plates), both from SA Biosciences, were then used to amplify muscle specific gene transcripts from cDNA using a 7300 ABI PCR machine. Data was analyzed using the software provided by the manufacturer. Because the gene expression of the five housekeeping genes on the PCR Array plates changed in response to cardiotoxin treatment, we normalized gene expression to histone deacetylase 5, which was not altered in response to cardiotoxin treatment, or between WT and Hsp70$^{-/-}$ muscles during either condition.
Western Blot Analyses

Skeletal muscle protein extracts were prepared from gastrocnemius muscles from WT and Hsp70−/− mice. Briefly, muscles were homogenized using a Dounce homogenizer in 1:10 (wt/vol) RIPA buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% SDS, 0.5% Na Deoxycholate) plus protease inhibitor cocktail and phosphatase inhibitors (Sigma Aldrich). Homogenates were centrifuged at 5000g for 15 min at 4°C and the supernatant collected. Protein content was assessed from supernatants using Bio-Rad DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (30µg) were separated for 45 minutes on 4-15% gradient polyacrylamide gels containing 0.1% SDS using electrophoresis at 200V at room temperature. Separated proteins were electrotransferred to polyvinylidene fluoride membranes for 90 minutes at 100V in Tris-Glycine buffer containing methanol at 4°C. To prevent non-specific binding, membranes were subsequently blocked in PBS-tween (PBS-T) containing 5% milk at room temperature for 1 hour. Membranes were incubated in primary antibody diluted in blocking buffer (Hsp70, 1:500, Stressgen) overnight at 4°C with gentle rocking. The next day membranes were washed extensively with PBS-T followed by incubation in secondary antibody (LI-COR Biosciences) for 1 hour at room temperature. Following extensive washing in PBS-T, membranes were rinsed in PBS and visualized using an infrared imaging system to detect and analyze labeled proteins (LI-COR Biosciences).

In vivo, Muscle Transfection and Rescue Experiments

The Hsp70-EGFP expression plasmid was created and used previously by our lab group (80). The EGFP expression plasmid was purchased from Clonetech. In vivo plasmid DNA injection and electroporation has been described previously by our lab group (81). Briefly, 10 µg of Hsp70-EGFP or EGFP dissolved in 25µl PBS was injected
along the longitudinal axis of the TA muscle using a 28 gauge insulin syringe. One minute following injection, five electric pulses at 75 V/cm, duration of 20 ms, and interpulse interval of 200 ms were delivered to the muscle using an electric pulse generator (Electro Square Porator ECM 830, BTX), by placing two paddle-like electrodes on either side of the muscle. Following experimental treatments and muscle harvest, EGFP and Hsp70-EGFP fluorescence was visualized in muscle cross-sections using a Leica DM5000B microscope and a GFP filter.

**Statistical Analyses**

All data were analyzed using ANOVA followed by Tukey’s post-hoc comparisons or a Student’s t-test when appropriate (GraphPad Software, San Diego, CA, USA). All data are expressed as means ± SE, and significance was established at p ≤ 0.05.
CHAPTER 3
RESULTS AND DISCUSSION

Genetic Deletion of Hsp70 Impairs Skeletal Muscle Fiber Growth

The current study is the first to determine the effect of Hsp70 knockout on skeletal muscle plasticity. Although we are specifically interested in the requirement of Hsp70 in regulating plasticity in post-natal skeletal muscle, to our knowledge, the phenotype of muscles which lack Hsp70 during embryonic growth and development has not been determined. Therefore, the use of lifelong Hsp70⁻/⁻ mice in the current study also allowed us to determine whether knockout of Hsp70 during development results in an overt muscle phenotype. Confirmation that Hsp70⁻/⁻ mice do not express Hsp70 was verified through qRT-PCR (Figure 3-1 A) on gastrocnemius muscles from WT and Hsp70⁻/⁻ mice using primers for Hspa1a and Hspa1b gene transcripts, which both code for Hsp70. Western blot analyses using an Hsp70 antibody was also performed to confirm the absence of Hsp70 protein (Figure 3-1 B), using tubulin as a loading control.

To determine whether Hsp70⁻/⁻ mice display normal muscle phenotype, tibialis anterior (TA) muscles from 4-week old WT and Hsp70⁻/⁻ mice were removed and processed for H&E analyses. As depicted in Figure 3-2, skeletal muscles from WT mice (A) and Hsp70⁻/⁻ mice (B) were morphologically indistinguishable at 4 weeks of age on H&E stain. Measurement of skeletal muscle fiber cross-sectional area and total numbers of muscle fibers in entire TA cross-sections from WT mice and Hsp70⁻/⁻ mice revealed no significant differences, (data not shown). These data indicate that Hsp70 is dispensable for normal embryonic skeletal muscle developmental and early post-natal skeletal muscle growth, at least up until 4 weeks of age.
Since post-natal muscle growth and maturation in mice continues until approximately 12 weeks of age, we further compared fiber cross-sectional area and fiber-type composition in muscles from 12-week old WT and Hsp70/− mice. To do this, we immunostained muscle cross-sections to identify Type I, Type IIa and Type IIb/x muscle fibers and measured CSA for each fiber type in entire cross-sections, n= at least 4 mice per group (Figure 3-3). This allowed us to determine fiber type composition, total number of muscle fibers, and average CSA for each fiber type. Although the soleus muscle is a predominately slow muscle, we chose to measure these variables in the soleus since it contains all three fiber types, which are distinguished based on the relative isoform of myosin heavy chain expressed in the muscle fiber. WT mice had average CSAs of 1457 μm² for Type I fibers, 1307 μm² for Type IIa fibers and 2000 μm² for Type IIb/x fibers, with an average fiber CSA of 1,588 μm² across all muscle fibers. Soleus muscles from Hsp70/− mice had average CSAs of 1157 μm² for Type I fibers (21% smaller than WT, p<0.05), 1063 μm² for Type IIa fibers (19% smaller than WT, p<0.05) and 1549 μm² for Type IIb/x fibers (23% smaller than WT, p<0.05), with an average fiber CSA of 1256 μm² across all muscle fibers (17% smaller than WT, p<0.05) (Figure 3-3 B). The average number of fibers for each fiber type in soleus muscles was not statistically different between WT and Hsp70/− mice (Figure 3-3 C). Therefore, fiber type composition was not altered in Hsp70/− mice.

Together these data indicate that while Hsp70 is dispensable for normal skeletal muscle development and post-natal muscle growth up until 4 weeks of age, Hsp70 appears to play an important role in regulating skeletal muscle fiber size. This discrepancy in muscle fiber size between WT and Hsp70/− mice may reflect deficits in
the later stage of skeletal muscle growth and maturation that occurs between 4 and 12 weeks of age in mice. However, since we have previously demonstrated that Hsp70 overexpression represses atrophy-related signaling pathways in skeletal muscle, the decreased skeletal muscle fiber CSA in Hsp70−/− mice may be due to increased signaling through these atrophic pathways in the absence of Hsp70, even under normal physiological conditions. Although the precise mechanism to explain this deficit in fiber size is not clear, these data demonstrate that Hsp70 is necessary for normal skeletal muscle fiber size under normal physiological conditions.

**Hsp70−/− Mice have Impaired Muscle Regeneration**

In order to determine the requirement of Hsp70 for normal muscle regeneration and recovery following injury, we injected TA muscles of 7 week-old WT and Hsp70−/− mice with 100 μl of 10 μM cardiotoxin, which is known to result in widespread muscle fiber necrosis. This standardized method of muscle injury allows for the study of muscle fiber degeneration and subsequent muscle fiber regeneration. Muscles were harvested 1, 4, 16, 28 or 42 days following cardiotoxin injury. Analyses of muscle cross-sections one day post-injury via H&E demonstrate that TA muscles from WT mice showed signs of edema, inflammatory cell infiltration and myofiber necrosis, as indicated by irregular eosinophilic staining of fibers with loss of normal fiber architecture (Figure 3-4 A). Four days post-cardiotoxin, necrotic fibers were largely cleared in WT muscles and replaced by mononuclear cells and central nucleated myoblasts, indicative of the early phase of muscle regeneration (Figure 3-4 B). In contrast, injured muscles from Hsp70−/− mice one day post-cardiotoxin showed less overt signs of fiber damage than WT, as visualized by fewer numbers of inflammatory cell infiltrates and preservation of fibers with normal
eosinophilic staining and fiber borders. However, despite this apparent decrease in
muscle fiber damage one day post-cardiotoxin, Hsp70\(^{-/+}\) animals displayed marked
abnormalities in morphology four days post-cardiotoxin, including persisting necrotic
fibers with abnormal basophilic staining which appeared reminiscent of myofiber
calcifications (8). H&E analysis of injured TA muscles from WT mice 16 days post-
cardiotoxin revealed restoration of normal muscle fiber architecture and centrally
nucleated regenerating muscle fibers (Figure 3-5 A). In contrast, TA muscles from
Hsp70\(^{-/-}\) mice still presented with numerous necrotic muscle fibers and inflammatory
lesions at this time point, and similarly displayed signs of myofiber calcifications seen in
the Hsp70\(^{-/-}\) mice four days post-cardiotoxin (Figure 3-5 B). Importantly, as shown in
Figure 3-5 C the average cross-sectional area of regenerating muscle fibers (those
containing centralized nuclei) in Hsp70\(^{-/-}\) TA muscles (945 \(\mu\text{m}^2\)) was significantly smaller
than that of WT (1524 \(\mu\text{m}^2\), n=4 muscles/group, \(p<0.05\). Furthermore, the average
number of regenerating myofibers containing centralized nuclei in injured muscles was
nearly 40% greater in Hsp70\(^{-/-}\) mice when compared to WT mice at this time point
(Figure 3-5 D). Presentation of this data as a frequency distribution (Figure 3-5 E)
demonstrates that the increased numbers of regenerating fibers in muscles from
Hsp70\(^{-/-}\) mice lie predominately in the CSA range of 250-750 \(\mu\text{m}^2\) (bin center = 500).
This significant increase in small regenerating fibers in muscles from Hsp70\(^{-/-}\) animals at
this time point could be the result of ongoing cycles of muscle degeneration/
regeneration. However, this finding is also suggestive of decreased myotube fusion,
since the muscles from Hsp70\(^{-/-}\) mice also had significantly lower numbers of fibers
containing centralized nuclei in the larger CSA range (>1500 \(\mu\text{m}^2\)).
Persistent Muscle Inflammation in Hsp70<sup>−/−</sup> Mice following Injury

Sustained and elevated muscle inflammation is well-evidenced to interfere with muscle regeneration (1, 91). Based on the persisting presence of mononuclear cells and necrotic muscle fibers seen in H&E stained sections from Hsp70<sup>−/−</sup> muscles 16 days post-cardiotoxin, we determined whether the Hsp70<sup>−/−</sup> mice indeed showed increased markers of inflammation. To do this we stained for the macrophage cell surface marker, CD68, which is a transmembrane glycoprotein expressed on macrophages with a pro-inflammatory (M1) phenotype. Serial sections from WT and Hsp70<sup>−/−</sup> muscles 16 days post cardiotoxin-injury were incubated with antibodies for CD68 (green) and laminin (red), which stains the basement membrane of muscle fibers. As shown in Figure 3-6 A, significant CD68 positive staining was seen across entire muscle cross-sections from Hsp70<sup>−/−</sup>, but not WT mice. Magnification and separation of the overlayed images to show CD68 and laminin staining separately in Figure 3-6 B, further demonstrates that the CD68-positive macrophages are present both inside and surrounding muscle fibers. Comparison of these CD68-stained areas to serial sections stained with H&E further demonstrate that the abnormal basophilic-stained fibers seen in the Hsp70<sup>−/−</sup> mice are also positive for macrophages (corresponding fibers in CD68- and H&E-stained sections are indicated with arrows). Further, fibers which appear infiltrated by numerous mononuclear cells (basophilic puncti) on H&E stain (outlined in white) also stain diffusely for CD68, demonstrating that these necrotic fibers are strongly infiltrated by pro-inflammatory macrophages. The average number of CD68 positive cells (outside the basal lamina) and the average number of fibers which show positive staining for CD68-positive macrophages (inside the basal lamina) were quantified separately in entire muscle cross-sections in WT and Hsp70<sup>−/−</sup> mice 16 days post-injury (Figure 3-6 C.
and D). Injured muscles from Hsp70<sup>−/−</sup> mice showed an approximate 7-fold increase in both the number of CD68-positive macrophages surrounding muscle fibers, and the number of muscle fibers infiltrated by CD68-positive macrophages, which further demonstrates the magnitude of inflammation still present 2 weeks post-injury in muscles lacking Hsp70. These data suggest that Hsp70 is necessary for both normal muscle regeneration and resolution of the immune response following muscle injury.

**Calcification of Regenerating Muscle from Hsp70<sup>−/−</sup> Mice following Injury**

Following damage to the muscle membrane as a result of muscle injury, intracellular calcium concentrations rise due to an influx of extracellular calcium (97). The cardiotoxin-induced model of muscle regeneration is believed to induce muscle injury through disrupting calcium homeostasis, causing depolarization and contracture of muscle fibers which results in mechanical injury to the muscle (43). Further, increased levels of intracellular calcium also play important roles in initiating muscle fiber proteolytic pathways and degeneration (97), though calcium can also activate membrane repair processes. Importantly, this excess calcium is eventually cleared during the normal cycle of degeneration/regeneration and calcium deposition does not occur. Based on the abnormal basophilic staining of muscle fibers in regenerating muscles from Hsp70<sup>−/−</sup> mice which appeared similar to muscle fibers staining positive for calcium deposits in previous studies (Yi Ping Li), we determined whether these abnormal fibers were positive on Von Kossa stain, which stains calcium in black. Indeed, as visualized through Von Kossa staining, which stains calcium deposits black, WT muscles do not show calcium deposition 16 days post-injury (Figure 3-7 A). In contrast, muscles from Hsp70<sup>−/−</sup> mice show pronounced calcium deposition in injured muscles 16 days post-injury (Figure 7 A and B). When compared to an H&E-stained
serial section from the same area of the muscle, it is clear that these calcium deposits are associated with inflammatory lesions and abnormal basophilic staining of fibers that in earlier experiments also stained positive for CD68+ macrophages. Increased levels of calcium and calcium deposits are a hallmark of persistent muscle degeneration and fiber necrosis that is seen in dystrophic muscle phenotypes (97). Between the deficits in regenerating muscle fiber size, sustained presence of pro-inflammatory macrophages and development of calcifications following injury, these data clearly demonstrate that mice lacking Hsp70 have significant impairments in muscle regeneration and recovery following injury.

Muscles from Hsp70−/− Mice Show Altered Gene-Expression Profile 4 Days Post Cardiotoxin-Injury

The impairments in muscle regeneration seen in muscles of Hsp70−/− mice 16 days post-cardiotoxin may result from impairments in various aspects of the muscle degeneration/regeneration process. Since marked abnormalities were noted in morphology as early as 4 days post cardiotoxin-injury, we chose to compare the gene expression changes in response to cardiotoxin in muscles from WT and Hsp70−/− mice 4 days post-injury. Since cardiotoxin-injury of the muscle is localized to the area of injection, the uninjured, contralateral TA muscle was used as an internal control for each animal (n=3 muscles/group). The gene expression profile of regenerating skeletal muscles following cardiotoxin injury has previously been demonstrated (100). Various genes are coordinately regulated during muscle regeneration, including genes involved cell cycle control, inflammatory cytokines, matrix remodeling proteins and muscle-specific transcription factors that are involved in muscle satellite cell activation and differentiation (100). We chose to utilize Myogenesis and Myopathy PCR arrays to
compare the relative changes in gene expression in response to cardiotoxin in WT and Hsp70\(^{-/-}\) animals. These arrays allowed for the measurement of gene expression changes of 84 genes known to be differentially regulated in skeletal muscle during myopathy and muscle regeneration. Since all housekeeping genes on the PCR arrays changed in response to cardiotoxin, we chose to normalize gene expression to HDAC5, whose gene expression levels did not significantly differ between WT and Hsp70\(^{-/-}\) mice in uninjured TAs, or in response to cardiotoxin. When comparing the magnitude of change in these genes between WT and Hsp70\(^{-/-}\) mice, numerous genes showed significantly higher magnitudes of activation in response to cardiotoxin in muscles from Hsp70\(^{-/-}\) mice (Figure 3-8 A-C). Among these genes were pro-inflammatory cytokines TNF\(\alpha\) (13-fold vs. 40-fold, p<0.05) and IL-6 (6-fold vs. 14-fold), growth factor IGF-II (68-fold vs. 147-fold, p<0.05), the extracellular matrix remodeling protein, MMP-9 (21-fold vs 60-fold, p<0.05), and the calcium-activated proteases, Calpain 2 (3-fold vs. 5-fold, p<0.05), Calpain 3 (0.9-fold vs. 2-fold, p<0.05) and Caspase 3 (19-fold vs. 32-fold, p<0.05). Similarly, despite the deficits in regenerating fiber size seen in the Hsp70\(^{-/-}\) TA muscles 16 days post-cardiotoxin, many of the typical markers of satellite cell activation, proliferation and differentiation in the Hsp70\(^{-/-}\) mice were either elevated or unaltered compared to WT. The magnitudes of activation when comparing WT vs. Hsp70\(^{-/-}\) mice were as follows, Myogenin (103-fold vs 259-fold, p <0.05), Myf5 (28-fold vs 40-fold) Troponin C (118-fold vs 401-fold, p<0.05) and Troponin T1 (58-fold vs 215-fold, p<0.05). Although Troponin C and Troponin T1 are typically thought as proteins expressed in adult slow muscle fibers, they are also highly expressed in myoblasts differentiating into myotubes (65). Other myogenic markers including MyoD, Pax3 and
Pax7 were not altered in Hsp70\(^{-/}\) mice when compared to WT. To summarize, injured muscles from Hsp70\(^{-/}\) mice showed elevations in numerous proteins involved in muscle fiber degeneration and necrosis, inflammation as well as those which promote regeneration. This quantitative gene data supports our histological findings in injured muscles from Hsp70\(^{-/}\) mice which showed evidence for increased inflammation and pro-inflammatory macrophages, which are known to secrete both pro-inflammatory cytokines and growth factors. Similarly, increased gene expression of the calcium-regulated proteases involved in muscle protein breakdown in regenerating muscles from Hsp70\(^{-/}\) mice is in line with our findings of calcium deposits that were strongly associated with degenerating myofibers and inflammatory lesions in these mice. In contrast, our finding that Hsp70\(^{-/}\) mice have either increased or unaltered levels of proteins involved in activating the myogenic program was unexpected, as our histological findings 16 days post-injury demonstrate that Hsp70\(^{-/}\) mice have deficits in muscle regeneration.

**Muscles from Hsp70\(^{-/}\) Mice do not Show Deficits in Cellular Proliferation following Injury**

As many of the markers of satellite cell activation and proliferation were either unaltered or increased in Hsp70\(^{-/}\) mice compared to WT, this suggests that Hsp70\(^{-/}\) mice are not compromised in their ability to increase satellite cell proliferation following injury. To further test this, WT and Hsp70\(^{-/}\) mice were injected IP with BrdU daily following cardiotoxin-injury, to label proliferating cells in regenerating muscles. Six days post-injury, the injured TA and the contralateral, uninjured TA were harvested for histological analyses. Muscle cross-sections from injured and uninjured TA muscles were co-stained with antibodies for BrdU and laminin, which stains the muscle fiber
basement membrane (Figure 3-9). Cross-sections from injured TA muscles showing positive BrdU staining were also counterstained with Dapi, to confirm the nuclear location of BrdU. Uninjured muscles from WT and Hsp70 mice showed relatively few BrdU-positive nuclei. In contrast, injured muscles from WT and Hsp70−/− animals showed abundant levels of BrdU nuclear staining in the center of muscle fibers, which is indicative of satellite cell proliferation and differentiation (Figure 3-9 B). Although some BrdU positive nuclei may be those of inflammatory cells, endothelial cells or other proliferating cells present in skeletal muscle, the consistent location of these BrdU positive nuclei in the center of regenerating myofibers outlined by laminin strongly suggests that many of these nuclei indeed were derived from satellite cell proliferation. Therefore, our findings using BrdU-labeling of proliferating cells are in agreement with our gene expression data, which indicate that Hsp70−/− mice do have deficits in satellite cell proliferation and differentiation, despite the deficits in regenerating fiber size in these mice two weeks post-injury.

Based on the significant elevation in the expression of the pro-inflammatory cytokine, TNFα, in cardiotoxin-injured muscles from Hsp70−/− mice, and the persisting levels of pro-inflammatory macrophages in these muscles 16 days post-injury, the deficits in muscle regeneration in Hsp70−/− animals may be related to impairments in resolving the pro-inflammatory immune response. Indeed, persisting levels of TNF-α and other pro-inflammatory cytokines known to be released by pro-inflammatory macrophages have inhibitory effects on muscle regeneration and recovery. Furthermore, since injured muscles from Hsp70−/− mice also developed calcium deposits following injury, this indicates that these muscles may have ongoing calcium-regulated
proteolysis and fiber degeneration, which is supported by our histological findings 16 days post-injury. Therefore, ongoing muscle fiber degeneration in mice lacking Hsp70 may also interfere with successful regeneration.

**Muscles from Hsp70<sup>-/-</sup> Show Impaired Muscle Recovery up to 6-weeks Post-Injury**

Persistent levels of inflammation and failed regeneration in skeletal muscle is known to result in replacement of muscle tissue with fibrotic tissue. Based on our findings that TA muscles from Hsp70<sup>-/-</sup> mice recovering from cardiotoxin-injury show much higher levels of pro-inflammatory macrophages, calcifications and smaller regenerating fibers we hypothesized that these muscles would also show increased collagen deposition, which results in fibrosis. Trichrome staining of muscle cross-sections 16 and 28 days post cardiotoxin-injury, which stains collagen blue, indeed demonstrates increased collagen deposition around muscle fibers in Hsp70<sup>-/-</sup> muscles when compared to WT at both time points (Figure 3-10 A and B).

Quantitative analyses of fiber cross-sectional area 28 days and 42 days post-injury in WT and Hsp70<sup>-/-</sup> mice provide further evidence for the failed recovery of muscles following injury in the absence of Hsp70 (Figure 3-11). Indeed, at both of these time points, muscle fibers from Hsp70<sup>-/-</sup> mice were still significantly smaller than those of WT (28 days post-injury, WT = 2130 µm<sup>2</sup> and Hsp70<sup>-/-</sup> = 1251µm<sup>2</sup>, p<0.05; 42 days post-injury, WT = 1754 µm<sup>2</sup> and Hsp70<sup>-/-</sup> = 1461µm<sup>2</sup>, p<0.05). Morphological analyses of muscles at the 42 day time point post-injury through H&E staining further demonstrated that muscles from Hsp70<sup>-/-</sup> mice still displayed areas of inflammation, signs of fibrosis and calcium deposition, and clear deficits in fiber size.
At all time points measured post-injury, regenerating myofibers in Hsp70<sup>−/−</sup> mice did not re-grow to a comparable size to regenerating myofibers in WT mice. These data therefore indicate that Hsp70<sup>−/−</sup> mice have impairments in the processes which allow for normal muscle fiber regeneration and re-growth following muscle injury. Muscle regeneration and growth following injury is a multistep process which involves satellite cell activation, proliferation, differentiation and fusion of myoblasts to regenerate multinucleated myofibers (7). Since Hsp70<sup>−/−</sup> mice did not show deficits in the typical markers of satellite cell activation, proliferation and differentiation following injury, these data indicate that muscles from Hsp70<sup>−/−</sup> mice may have deficits in the later phases of muscle regeneration that allow for myofiber growth. Importantly, fusion of differentiated myoblasts is an important event which both augments myofiber size and subsequently decreases fiber number. Since Hsp70<sup>−/−</sup> mice also showed increased numbers of regenerating myofibers, in addition to decreased CSA of these regenerating fibers, we further suspect that Hsp70<sup>−/−</sup> mice may have deficits in myotube fusion. Although the detailed experiments needed to confirm this hypothesis are beyond the scope of the current study, this certainly warrants further investigation.

**Muscle Regrowth and Regeneration following Reloading-induced Injury is Impaired in Hsp70<sup>−/−</sup> Mice**

While our cardiotoxin studies clearly demonstrate that Hsp70<sup>−/−</sup> mice have deficits in muscle regeneration and recovery following muscle injury, we sought to determine whether this would hold true in response to a more physiologically relevant form of muscle injury. One model of physiological muscle injury is that of muscle reloading injury (also referred to as modified muscle use), which occurs during the reambulation period following a prolonged period of muscle unloading or muscle disuse (17). For
reasons largely unknown, following muscle disuse due to hind limb immobilization or hind limb suspension, although the soleus, plantaris and gastrocnemius muscles all atrophy, upon reambulation only the soleus muscle demonstrates significant reloading-damage (17). We therefore cast-immobilized the hind limbs of WT and Hsp70/− mice for 10 days, removed the casts and allowed the mice to reambulate for either 0, 3 or 10 days prior to tissue harvest. Muscle morphology was assessed at each time point through H&E staining of muscle cross-sections (Figure 3-12 A). Immediately following cast-removal (0 days reloading) soleus muscles from neither WT nor Hsp70/− mice showed evidence of muscle damage. However, following 3 days of skeletal muscle reloading, WT and Hsp70/− muscles showed evidence of edema, inflammatory cell infiltration and muscle fiber necrosis. Early stage necrotic fibers, as identified on H&E stain as those fibers containing reduced eosinophilic staining with inflammatory cells infiltrating or surrounding the fiber, were minimally present in WT muscles (Figure 3-12 B). Necrotic fibers in WT muscles were either in late-stage necrosis (heavily infiltrated by inflammatory cells and devoid of clear fiber borders) or cleared and replaced by mononuclear cells. However, in muscles from Hsp70/− mice, these early stage necrotic fibers were visually increased, and quantified to be ~7-fold higher than in WT muscles (Figure 3-12 B), n = 4 mice per group, p<0.05. Although this may be interpreted such that muscles from Hsp70/− mice show increased muscle-reloading damage, this did not appear to be the case. Indeed, despite the increased numbers of necrotic fibers in the 3-day reloaded Hsp70/− muscles, there were also more intact muscle fibers remaining at this time-point in the Hsp70/− mice when compared to WT. We hypothesize that muscles from Hsp70/− mice have a delayed and/or impaired muscle injury response,
thereby resulting in delayed appearance and subsequent clearance of necrotic muscle fibers. Importantly, following 10 days of muscle reloading, similar to our findings using cardiotoxin-injury, muscles from WT mice had largely regained normal muscle fiber architecture and displayed many centrally nucleated fibers, while damaged areas from Hsp70\(^{-/-}\) mice were not recovered. Indeed, 10-day reloaded muscles from Hsp70\(^{-/-}\) mice showed abnormal muscle fiber architecture consisting of small rounded fibers, hypercontracted or swollen muscle fibers (indicated by white arrow in the bottom panel of Figure 3-12 A) and increased space between individual muscle fibers. Although the average number of fibers containing centralized nuclei in 10-day reloaded muscles were much lower in Hsp70\(^{-/-}\) mice when compared to WT (Figure 3-12 C), quantification of fiber size in those fibers which did contain centralized nuclei revealed regenerating fibers in muscles from Hsp70\(^{-/-}\) mice were significantly smaller than those from WT muscles (Figure 3-12 D). Representation of the CSA of regenerating fibers as a frequency distribution further demonstrates a significant left-shift in the Hsp70\(^{-/-}\) mice, showing increased numbers of small, regenerating fibers, and decreased numbers of larger, regenerating fibers (Figure 3-12 E).

Since we found in our cardiotoxin experiments that injured muscles from Hsp70\(^{-/-}\) mice showed elevations in both inflammatory and regenerative markers when compared to WT, we determined whether this would similarly be true in response to reloading injury. To assess inflammation, cross-sections from 3-day reloaded muscles were stained with antibodies for the pro-inflammatory macrophage marker CD68 (green-fluorescence) and laminin (red fluorescence), which stains muscle fiber basement membranes. Representative cross-sections shown in Figure 3-13 demonstrates that WT
and Hsp70−/− muscles both showed significant levels of CD68-positive macrophages at this time point. Cross-sections from 3-day reloaded muscles were also stained with embryonic myosin heavy chain (eMHC) (red fluorescence), which is transcribed by de novo formed myonuclei following satellite cell activation and differentiation, to assess the relative levels of regeneration in muscles at this time point (Figure 3-13 B). Co-staining with dapi, which stains nuclei blue, helped to visualize the eMHC-positive myoblasts and myofibers. Of note, muscles from WT mice showed numerous eMHC-positive myoblasts, but few EMHC-positive myofibers at this time point. In contrast, Hsp70−/− mice showed numerous eMHC-positive myofibers. Analysis of gene expression in muscles following 3 days of muscle reloading allowed for a more quantitative measurement of inflammatory and regenerative markers in WT and Hsp70−/− mice, and is shown in Figure 3-14. Muscles from Hsp70−/− mice demonstrated significant elevations in the mRNA levels of regenerative markers MyoD and myogenin, and inflammatory markers CD68 and TNF-α when compared to WT in 3-day reloaded muscles. Hsp70−/− mice also showed increased mRNA levels of the prototypical NF-κB family member, p65, which is involved in classical NF-κB activation and pro-inflammatory signaling. Interestingly, iNOS, which is often used as a marker of skeletal muscle damage due to its role in NO-mediated damage of muscle fiber membranes, was significantly decreased in muscles from Hsp70−/− mice under control conditions, and its activation was similarly blunted in 3-day reloaded muscles. Inhibition of nitric oxide production following muscle injury was recently shown to interfere with muscle regeneration and increase collagen deposition (15). Therefore, decreased iNOS and nitric oxide signaling in Hsp70−/− mice could play a role in both the decreased and/or delayed muscle injury
response observed in Hsp70\(^{-/-}\) mice in the first few days following injury, as well as the impairments in muscle regeneration and recovery seen in these mice at later time points.

Data collected thus far demonstrate that Hsp70\(^{-/-}\) mice indeed have deficits in muscle regeneration and recovery following two distinct modes of muscle injury, cardiotoxin-induced injury and mechanical injury due to modified muscle use. In response to both experimental models of muscle injury, muscles from Hsp70\(^{-/-}\) mice had elevations in inflammatory processes and defects in muscle recovery. Importantly, these deficits in muscle recovery included decreased size of regenerating myofibers. However, muscles from Hsp70\(^{-/-}\) mice did not show deficits in the normal regenerative markers during the early phase of muscle regeneration, including BrdU incorporation as a measure of proliferative capacity, MyoD and myogenin mRNA levels, and eMHC expression. Therefore, the deficits in regenerating fiber size in Hsp70\(^{-/-}\) mice may instead be related to deregulation of the inflammatory response which may subsequently impair fiber regeneration. Terminal differentiation and growth of regenerating fibers in the later phase of muscle regeneration involves fusion of myoblasts to create multinucleated myotubes. Importantly, fusion of myoblasts can be repressed by pro-inflammatory cytokines, such as TNF-\(\alpha\) and IL-6 (37, 38, 98), two cytokines which were elevated in injured muscles from Hsp70\(^{-/-}\) mice above WT. Further, pro-inflammatory macrophages are a major source of these cytokines in injured muscle, and Hsp70\(^{-/-}\) mice showed elevations in these pro-inflammatory macrophages up to 16 days post-injury. Therefore it seems plausible that sustained inflammation in muscles of Hsp70\(^{-/-}\) mice may contribute to the deficits in regenerating fiber size through
inhibiting myoblast fusion. Importantly TNF-α and IL-6 also have positive effects on muscle regeneration through their proproliferative action on satellite cells in the early days following muscle injury (41, 98). Therefore, the increased expression of TNF-α and IL-6 in injured muscles 4 days post-injury in Hsp70−/− mice may also explain the elevations in regenerative markers seen in these mice at this time point.

Interestingly, iNOS-mediated production of NO from muscle fibroblasts was recently shown to promote myoblast fusion during post-natal skeletal muscle growth and maturation (12). Since we saw significant reductions in iNOS mRNA in muscles from Hsp70−/− mice during both control conditions and following muscle injury, it may be speculated that decreased NO signaling (and therefore decreased myoblast fusion) may contribute to the increased numbers of myofibers with smaller CSA in regenerating muscles from these mice.

**Restoration of Hsp70 in Skeletal Muscle Restores Regenerative Deficits in Hsp70−/− Mice**

Since Hsp70 whole-body knockout mice were used in the current study, the deficits in muscle regeneration and recovery may be due to the lack of Hsp70 in any cell type involved in muscle regeneration, including muscle fibers, satellite cells, endothelial cells, fibroblasts, inflammatory cells and other cells residing in skeletal muscle tissue. To determine whether restoration of Hsp70 levels in skeletal muscle fibers specifically can prevent the deficits in regeneration in the Hsp70−/− mice, we performed rescue experiments via plasmid injection and electroporation of skeletal muscle with Hsp70-EGFP or EGFP expression plasmids. This method has been used extensively by numerous lab groups to induce transgene expression specifically in skeletal muscle fibers (70, 75, 80, 95). Hsp70-EGFP or EGFP plasmids were injected and
electroporated into Hsp70\(^{-/-}\) TA muscles either 4 days prior to cardiotoxin injection or 4 days post-cardiotoxin injection. As we observed abnormal gene expression and morphology in Hsp70\(^{-/-}\) mice as early as 4 days post-cardiotoxin, this suggested to us that Hsp70\(^{-/-}\) mice may have significant impairments in the coordinated events which occur during the first few days following muscle injury that allow for successful regeneration. Therefore, electroporation of Hsp70-EGFP into the muscle prior to cardiotoxin-injury aimed to test the importance of Hsp70 expressed in muscle fibers at the onset of muscle injury, on the regulation of muscle regeneration and recovery. In contrast, electroporation of Hsp70-EGFP 4 days following cardiotoxin injection allows for the transduction of newly regenerating myotubes and myofibers. Therefore, the importance of Hsp70 in newly regenerating muscle fibers can be assessed from the fourth day following injury and beyond. Comparisons on muscle recovery can then be made between Hsp70\(^{-/-}\) muscles transfected with Hsp70-GFP prior to cardiotoxin and following cardiotoxin injury to help delineate if and when muscle fiber-derived Hsp70 contributes to successful regeneration and recovery.

As shown in Figure 3-15, Hsp70\(^{-/-}\) muscles transfected with EGFP prior to cardiotoxin-injury still demonstrated heavy infiltration of inflammatory cells, signs of fibrosis and small regenerating fibers when visualized 16 days post-injury. In contrast, transfection of Hsp70\(^{-/-}\) muscles with Hsp70-EGFP prior to cardiotoxin-injury largely restored the regenerative deficits seen in the Hsp70\(^{-/-}\) muscles. These Hsp70-EGFP transfected muscles from Hsp70\(^{-/-}\) mice were almost completely devoid of inflammatory lesions and signs of fibrosis and contained visually larger regenerating fibers than Hsp70\(^{-/-}\) muscles transfected with EGFP.
Analyses of TA muscles that were transfected with Hsp70-EGFP (or EGFP) 4 days post-cardiotoxin, revealed only partial rescue to regenerating Hsp70-EGFP transfected muscle fibers (Figure 3-16). While Hsp70-EGFP positive fibers were visually larger than EGFP positive fibers rescued 4 days post-injury, numerous inflammatory lesions were still present throughout both the EGFP and Hsp70-EGFP transfected muscles. Therefore, the decrease in inflammatory cells seen in muscles transfected with Hsp70-EGFP prior to cardiotoxin injections was not visually apparent in muscles rescued with Hsp70-EGFP 4 days post-cardiotoxin.

Together these data indicate that muscle-derived Hsp70 is indeed necessary for normal muscle regeneration, and that Hsp70 plays an especially critical role during the first 4 days following cardiotoxin injury to promote a timely resolution of the inflammatory response. The release of muscle fiber-derived proteins to the extracellular space following membrane damage in the first few days following muscle injury is believed to play an important role in activating and recruiting inflammatory cells to the site of muscle injury (91). As Hsp70 has been shown in numerous studies to participate in both the innate and adaptive immune responses (84), based on our findings it seems plausible that Hsp70 and/or an Hsp70-chaperoned peptide released from injured muscle may participate in the immune response that proceeds muscle injury, which has been speculated on previously (42). Interestingly, Prakken et al demonstrated several years ago now that injection of recombinant Hsp70 protein into rats was sufficient to induce anti-inflammatory cytokines IL-10 and IL-4 in lymphatic cells, which is characteristic of a Th2 anti-inflammatory immune response (69). IL-10 and IL-4 are known to activate M2 macrophages (25), which play an important role in resolving the pro-inflammatory
microenvironment in injured muscle through deactivating M1 pro-inflammatory macrophages, and promoting muscle regeneration (91). Although we did not measure the levels of anti-inflammatory macrophages or cytokines in the current study, it may be speculated that the increased and sustained pro-inflammatory environment seen in muscles lacking Hsp70 are related to an imbalance in the M1/M2 macrophage phenotype. Further research to delineate the mechanisms whereby muscle-derived Hsp70 regulates the immune response is currently ongoing.
Figure 3-1. Confirmation of Hsp70 mRNA and protein knockout in Hsp70\textsuperscript{-/-} mice. A) qRT-PCR was performed on muscles from WT and Hsp70\textsuperscript{-/-} mice to confirm the absence of Hspa1a and Hspa1b gene transcripts, which code for Hsp70. B) Western blot analyses using an antibody for Hsp70 was further performed to confirm the absence of Hsp70 protein, using anti-tubulin as a loading control.
Figure 3-2. Lifelong Hsp70 knockout does not alter skeletal muscle development and morphology in 4-week old mice. A) Representative cross-sections of tibialis anterior (TA) muscles from 4-week old WT mice stained with H&E and visualized at high and low magnification to visualize individual muscle fibers and total muscle area. B) Representative cross-sections of TA muscles from 4-week old Hsp70⁻/⁻ mice stained with H&E and visualized at high and low magnification to visualize individual muscle fibers and total muscle area.
Figure 3-3. Decreased fiber cross-sectional area in soleus muscles of 12-week-old Hsp70 "−" mice. A) Representative soleus muscle cross-sections from 12-week old WT and Hsp70 "−" mice immunostained for Type I (blue), Type IIa (green) and Type IIb/x muscle fibers (black). B) Average fiber cross-sectional area (CSA) for Type I, IIa and IIb/x muscle fibers in soleus muscles from WT and Hsp70 "−" mice. C) Average number of Type I, IIa and IIb/x muscle fibers and total number of muscle fibers in soleus muscles from WT and Hsp70 "−" mice. All data represent mean ±SE, n=at least 4 mice/group, *p<0.05.
Figure 3-4. Abnormal morphology in Hsp70−/− TA muscles 4 days post cardiotoxin-injury. A) Representative H&E-stained cross-sections from cardiotoxin-injured TA muscles from WT and Hsp70−/− mice 1 day post-injury. B) Representative H&E-stained cross-sections from cardiotoxin-injured TA muscles from WT and Hsp70−/− mice 4 days post-injury.
Figure 3-5. Muscles from Hsp70−/− mice show deficits in regeneration 16 days post cardiotoxin-injury. A) Representative cross-section from injured WT TA muscle demonstrating numerous regenerating muscle fibers and restoration of normal muscle architecture 16 days post-injury. B) Representative cross-section from injured Hsp70−/− TA muscle showing impaired muscle recovery. C) Quantification of fiber CSA of fibers containing centralized nuclei (regenerating fibers) demonstrate that the CSA of regenerating fibers from Hsp70−/− TA muscles are significantly smaller than WT 16 days post-injury. D) Quantification of the average number of regenerating fibers per section (x20 field view) in WT and Hsp70−/− mice. E) Frequency distribution representing the percent of fibers at a given cross-sectional area (+/-250μm²), with the exception of the bin center 0, which contains fibers from 0-250μm². All data represent mean ±SE, n=4 muscles/group, *p<0.05.
Figure 3-6. Muscles from Hsp70−/− mice show increased numbers of CD68-positive macrophages 16 days post cardiotoxin-injury. A) Representative cross-sections from injured TA muscles from WT and Hsp70−/− mice 16 days post-injury stained with CD68 (green) to identify pro-inflammatory macrophages and laminin (red) to outline muscle fiber basement membranes. B) Magnification and separation of the merged images (boxed areas) in (A) to better demonstrate the localization of CD68-positive macrophages to areas surrounding and inside of the basal lamina. H&E staining of serial sections further confirms that muscle fibers heavily infiltrated by mononuclear cells on H&E-stain are also positive for CD68-positive macrophages. C) Quantification of the number of CD68-positive macrophages outside of the basal lamina. D) Quantification of the number of muscle fibers showing CD68-staining (macrophages) inside the muscle fiber. All data represent mean ±SE, n=4 muscles/group, *p<0.05.
Figure 3-7. Hsp70\(^{-/-}\) mice develop calcifications in muscles following cardiotoxin-injury. A) Representative Von Kossa stained cross-sections from cardiotoxin-injured WT and Hsp70\(^{-/-}\) TA muscles 16 days post-injury demonstrate the presence of calcium deposits in injured Hsp70\(^{-/-}\) but not WT muscles. B) Magnification of the Von Kossa-stained Hsp70\(^{-/-}\) TA cross-section (boxed area) is shown to better visualize the location of the deposits. An H&E-stained serial section from the same area of the muscle demonstrates that the deposits identified in Von Kossa stain are associated with the dark basophilic stained areas and inflammatory lesions see on H&E stain. Corresponding fibers in serial sections are indicated with asterisks.
Figure 3-8. Hsp70⁻/⁻ mice show altered gene expression 4 days post cardiotoxin-injury. Cardiotoxin-induced changes in gene expression 4 days post cardiotoxin-injury in WT and Hsp70⁻/⁻ muscles are grouped by their magnitude of activation. A) Gene expression changes under 5-fold. B) Gene expression changes between 5- and 50-fold. C) Gene expression changes greater than 50-fold. Injured muscles from Hsp70⁻/⁻ mice show enhanced activation of inflammatory cytokines, proteases, growth factors, calcium-regulated proteins and regenerative markers compared to WT. All data represent mean ±SE, n=3 muscles/group, *p<0.05.
Figure 3-9. Measurement of BrdU-positive proliferating cells in muscles from WT and Hsp70−/− mice following cardiotoxin-injury. A) Representative cross-sections from uninjured TA muscles injected with BrdU daily for 6 days, and immunostained for BrdU (red) and laminin (green). B) Representative cross-sections from injured TA muscles 6 days post-injury from WT and Hsp70−/− mice injected with BrdU daily beginning 6 hours prior to injury, and immunostained for BrdU (red) and laminin (green). Sections were also counterstained with DAPI (blue), to demonstrate co-labeling of BrdU with nuclei (pink) in merged images.
Figure 3-10. Muscles from Hsp70⁻/⁻ mice show increased collagen deposition post cardiotoxin-injury. Representative cross-sections from WT and Hsp70⁻/⁻ TA muscles 16 and 28 days post cardiotoxin-injury stained with Trichrome to identify collagen deposition (blue). A) 16 days post cardiotoxin-injury. B) 28 days post cardiotoxin-injury.
Figure 3-11. Regenerating muscles from Hsp70⁻/⁻ mice fail to recover up to 6 weeks post cardiotoxin-injury. A) Representative H&E-stained cross-sections of TA muscles from WT and Hsp70⁻/⁻ mice 42 days post cardiotoxin-injury. B) The average CSA of muscle fibers in WT and Hsp70⁻/⁻ TA muscles was calculated 28 days and 42 days post cardiotoxin-injury. Fibers from Hsp70⁻/⁻ TA muscles are significantly smaller than WT at both time points post-injury. All data represent mean ±SE, n=4 muscles/group, *p<0.05.
Figure 3-12. Muscles from Hsp70−/− mice show impaired regeneration and recovery following reloading injury. The hind limbs of WT and Hsp70−/− mice were cast-immobilized for 10 days, followed by cast-removal and reambulation for either 0, 3 or 10 days. A) Muscle morphology was visualized via H&E staining. B) Quantification of the average number of necrotic muscle fibers in 3-day reloaded muscles. C) Quantification of the average number of regenerating fibers containing centralized nuclei in 10-day reloaded muscles. D) Average CSA of regenerating fibers in 10-day reloaded muscles. E) Frequency distribution of regenerating fibers at a given CSA range in 10-day reloaded muscles. All data represent mean ±SE, n=4 mice/group, *p<0.05.
Figure 3-13. Inflammation and regeneration in soleus muscles of WT and Hsp70−/− mice following reloading-injury. WT and Hsp70−/− mice were exposed to 10 days of hind limb cast-immobilization followed by cast removal and 3 days of muscle reloading. A) Inflammation was assessed in muscle cross-sections through immunohistochemical staining for CD68+ macrophages (green) and laminin (red) which stains muscle fiber basement membranes. B) Muscle fiber regeneration was assessed via immunohistochemical staining for embryonic myosin heavy chain (EMHC) (red), which is expressed by regenerating myofibers and differentiating myoblasts. Sections were counterstained with Dapi to demonstrate that EMHC staining is localized to both small differentiating myoblasts and larger, regenerating myofibers.
Figure 3-14. Altered gene expression in muscles of Hsp70−/− mice following reloading injury. qRT-PCR was performed in gastrocnemius muscles from WT and Hsp70−/− mice exposed to 10 days of hind limb cast-immobilization followed by cast removal and 3 days of muscle reloading. A) Relative mRNA levels of myogenic marker, MyoD. B) Relative mRNA levels of myogenic marker, Myogenin. C) Relative mRNA levels of inflammatory marker, CD68. D) Relative mRNA levels of iNOS. E) Relative mRNA levels of pro-inflammatory cytokine TNF-α. F) Relative mRNA levels of the NF-κB transcription factor p65. All data represent mean ±SE, n=4 mice/group, *p<0.05.
Figure 3-15. Restoration of Hsp70 in muscle fibers 4 days prior to cardiotoxin-injury rescues deficits in regeneration and recovery in Hsp70−/− mice. TA muscles of Hsp70−/− mice were transfected with either EGFP or Hsp70-EGFP and 4 days later injected with cardiotoxin to induce muscle injury. A) Representative muscle cross-sections 16 days post cardiotoxin-injury showing EGFP and Hsp70-EGFP fluorescence under a GFP filter. B) Serial sections from injured muscles transfected with EGFP or Hsp70-EGFP prior to injury were stained with H&E to determine morphology 16 days post-injury.
Figure 3-16. Restoration of Hsp70 in regenerating muscle fibers of Hsp70<sup>−/−</sup> mice 4 days following cardiotoxin-injury visually increases regenerating fiber size. Muscles from Hsp70<sup>−/−</sup> mice were injected with cardiotoxin to induce muscle injury, and 4 days following injury, were transfected with EGFP or Hsp70-EGFP plasmids. A) Representative EGFP and Hsp70-EGFP fluorescence in muscle cross-sections 16 days post-injury as visualized under a GFP filter. B) Serial sections from injured muscles injected with EGFP or Hsp70-EGFP were stained with H&E to visualize muscle morphology. White asterisks denote corresponding muscle fibers in serial cross-sections.
CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

The current study used genetically modified mice which lack Hsp70 to test the hypothesis that Hsp70 is an indispensible protein necessary for normal skeletal muscle plasticity. We found that skeletal muscles from 4-week old Hsp70−/− mice were phenotypically similar to their WT counterparts, indicating that Hsp70 is not necessary for normal skeletal muscle development and early post-natal muscle growth. However, by 12-weeks of age, when skeletal muscle growth and maturation comes to an end, muscle fibers from Hsp70−/− mice were at this point significantly smaller in CSA when compared to WT. Therefore, these data suggest that Hsp70 may be necessary for the later stages of muscle growth and maturation. In addition, we also found that Hsp70−/− mice had significant deficits in muscle re-growth and recovery following muscle injury that persisted up to 6-weeks post-injury. These deficits in recovery in Hsp70−/− mice were characterized by smaller CSA of regenerating muscle fibers, enhanced and sustained muscle inflammation and necrosis, calcification of myofibers, and collagen deposition, which are all characteristics of a dystrophic muscle phenotype.

Successful muscle regeneration and re-growth of myofibers requires the activation and proliferation of satellite cells and their commitment to myogenic differentiation (7). Based on both gene expression analyses of proteins involved in satellite cell proliferation and differentiation and BrdU cellular proliferation assays post-injury, Hsp70−/− mice do not appear to have deficits in their ability to activate the myogenic program. However, successful growth of de novo myofibers not only requires satellite cell activation and differentiation, it also requires successful myoblast fusion to create the multi-nucleated myofibers that span the length of the muscle. Therefore, the deficits
in regenerating myofiber size seen in Hsp70−/− mice in the current study may be related to deficits in myoblast fusion. Although we did not quantitatively measure myoblast fusion in the current study, data collected in Hsp70−/− mice during rescue experiments support this notion. Indeed, the number of centralized nuclei per myofiber (which measures fusion index) in Hsp70-EGFP-positive myofibers appeared to be visually greater than that of EGFP-positive muscle fibers from Hsp70−/− TAs when visualized 16 days post-injury. Since myoblast fusion supports myofiber regrowth, and regenerating Hsp70-EGFP-positive myofibers were also visually larger than EGFP-positive myofibers at this time point, we hypothesize that Hsp70 supports myofiber growth in part, through promoting myoblast fusion. Although the detailed mechanisms to support this hypothesis were beyond the scope of the current study, this certainly warrants further study.

Importantly, in addition to the deficits in regenerating muscle fiber size, Hsp70−/− mice also showed significant elevations in pro-inflammatory markers persisted at numerous time points post-injury. While the specific mechanism behind the increased and sustained inflammatory signaling in regenerating muscles from Hsp70−/− mice was not defined in the current study, rescue experiments in which Hsp70 was restored in muscles prior to injury suggest that it may be related to impairments in the sequence of events which occur during the first 4 days following muscle injury. In this regard, rescue experiments in which Hsp70-EGFP was transfected into skeletal muscle fibers 4 days prior to cardiotoxin-injury prevented the inflammation and fiber necrosis seen in regenerating muscles of Hsp70−/− mice 16 days post-injury. In contrast, transfection of Hsp70-EGFP into muscles 4 days post cardiotoxin-injury did NOT prevent the
inflammation and fiber necrosis seen at this time point, despite the positive affect it had on increasing myofiber size. Therefore, we can conclude from these experiments that in addition to Hsp70 promoting growth of regenerating myofibers between days 4 and days 16 post-injury, Hsp70 expressed in muscle fibers at the time of muscle injury also promotes resolution of the inflammatory response through a mechanism which occurs during the first 4 days following muscle injury. It is well established that the pro-inflammatory immune response that occurs in skeletal muscle during the first few days following muscle injury is subsequently counteracted and resolved in part through a shift in the macrophage population towards an anti-inflammatory phenotype. These anti-inflammatory or M2 macrophages secrete anti-inflammatory, or Th2 cytokines which promote muscle regeneration and repair (91, 92). Interestingly, evidence exists to support Hsp70 as an activator of the Th2 immune response. Indeed, pre-immunization or injection of recombinant Hsp70 into the foot pads of mice is sufficient to induce an anti-inflammatory, or Th2 response in immune cells (69). Therefore, it may be speculated that Hsp70 and/or an Hsp70-chareroned peptide derived from injured muscle fibers may similarly promote an anti-inflammatory response in regenerating muscle following injury to promote muscle recovery. Future research to delineate the mechanisms in which skeletal muscle-derived Hsp70 regulates the immune response in regenerating muscle following muscle injury is currently underway.

In summary, findings from the current study demonstrate that Hsp70 is necessary for normal muscle regeneration and recovery. As experiments using muscle-specific Hsp70 transgenic mice have previously demonstrated that Hsp70 overexpression can enhance muscle regeneration and functional recovery following injury, the results from
this study provide further evidence to support the development and use of Hsp70-targeted therapeutics for a wide variety of skeletal muscle disorders in which muscle regeneration is compromised. Importantly, our findings suggest that induction of Hsp70 in skeletal muscle both prior to muscle injury and during the growth phase of muscle regeneration may optimally promote successful muscle regeneration and recovery.
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BIOGRAPHICAL SKETCH

Sarah Marie Senf graduated cum laude from the University of Florida with a Bachelor of Science degree in microbiology and cell science in December 2006. She then immediately went on to pursue a Ph.D. in exercise physiology from the University of Florida in January 2007. Sarah became a National Institute of Health T32 fellow in the Neuromuscular Plasticity Training Program at the University of Florida in 2008. Throughout her Ph.D. training, her research largely focused on understanding the molecular events which regulate skeletal muscle mass during atrophic conditions. Her early work demonstrating the protective role of Hsp70 on muscle mass during atrophic conditions led to her dissertation project which focused on the requirement of Hsp70 during skeletal muscle regeneration and recovery following injury.