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FoxO1 Induces Apoptosis in Skeletal Myotubes

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An Abstract of
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In treating ailments and pursuing rehabilitation, muscle atrophy is the most limiting factor effecting treatment efficiency^{22,34}. The loss of muscle mass due to aging, inactivity, injury and disease increases the likelihood for falls, loss of independence, and decrease in quality of life^{8,9,20,22,23,32,34-36,39,40,44}. Health care costs due to muscle atrophy run up to 18.5 billion (US) per year⁹. Due to the detrimental effect muscle atrophy has on both individuals and the economy; significant advances have been taken in understanding muscle atrophy and the various molecular and signaling pathways activated within the process^{8,9,23-25,34,35}. In our previous work with the muscle atrophy process we showed that FoxO1 activation promotes muscle atrophy evidenced through the decrease in protein content, which was accompanied by signs of apoptosis, namely DNA fragmentation. To test the hypothesis that FoxO1 activation promotes expression of genes associated with muscle atrophy and apoptosis and that this reaction is dependent upon FoxO1 DNA-binding, FoxO1-estrogen receptor fusion proteins (FoxO1^{AAA-ER} and FoxO1^{AAA/Arg215-ER} [DNA-binding deficient]) which are activated by treatment with 4-hydroxytamoxifen (4-

OHT) were stably transfected in C2C12 skeletal myoblasts using the pBABE retroviral system and grown into 4-day-old skeletal myotubes. Non-transfected C2C12 cells served as controls. After 24 hour treatment with vehicle or 4 OH-T, total RNA was isolated and gene expression performed using qPCR. The purpose of this study was to provide support for the phenotypic findings observed through assessing transcription activity of genes associated with muscle atrophy (Atrogin-1/MAFbx, Murf-1) and apoptosis (Bim, BNip3). Activation of FoxO1^{AAA-ER} resulted in a significant increase in Atrogin-1/MAFbx (~27 fold), Bim (~3.5 fold) and Murf-1 (~2 fold) gene expression, with no significant increase in BNip3 gene expression. Whereas, activation of the FoxO1^{AAA/Arg215-ER} resulted in a significant increase in Murf-1 (~2.2 fold), BNip3 (~2.2 fold) and Bim (~2 fold) gene expression, with no significant increase in Atrogin-1/MAFbx gene expression. No change in gene expression was observed in the control cells. These findings demonstrate that muscle atrophy induced via FoxO1 activation is associated with the induction of genes responsible for regulating protein degradation and apoptosis, via DNA binding dependent and independent mechanisms.

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Chapter One

Introduction

In treating ailments and pursuing rehabilitation, muscle atrophy is the most limiting factor effecting treatment efficiency^{22,34}. For example, sarcopenia, muscle atrophy due to aging, is prevalent in 10-20% of the population under the age of seventy. Likewise 40% of the population over eighty years of age experience significant muscle loss⁹. This rapid loss of muscle mass seen in aging as well as following injury, inactivity, and disease increases the likelihood of falls, loss of independence, and decrease in quality of life^{8,9,20,22,23,32,34-36,39,40,44}. Health care costs due to muscle atrophy run up to 18.5 billion (US) per year⁹. Hence, significant advances have been taken in understanding muscle atrophy using pharmacological and genetic manipulation in cells and rodents in order to understand, dampen and inhibit the molecular and signaling pathways that yield skeletal muscle atrophy^{8,9,23-25,34,35}.

Thus far research has shown that these atrophy inducing conditions each trigger different types of molecular and signaling pathways, however they all share the same outcome in muscle wasting^{8,20,22,23,34,35,44}. For example, immobilization can be caused by

numerous muscle atrophying conditions such as disease and injury or it can induce muscle atrophy in and of its self. The process causes morphological, physiological and biochemical alterations within skeletal muscle fibers that result in an imbalance of protein synthesis and breakdown. More specifically, immobilization activates the ubiquitin-proteasome pathway triggering major contractile protein degradation, noted by the decrease in cross sectional area, myofibers and force production. Immobilization has also shown to reduce myocyte number and decrease satellite cell activity, suggesting activation of apoptosis^{8,17,20, 39,44}. Other research supports that the decrease in muscle mass is attributed to cellular events involving protein degradation, in addition to mitochondrial dysfunction and loss of myonuclei^{8,17,25,30,35,39,44}. Currently, there are many different pathways which regulate muscle atrophy, however two of the more prominent pathways include protein ubiquitination and apoptosis^{8,10,17,22-25,29,34,35,39}.

Regulation of skeletal muscle mass is controlled primarily through the AKT/mTOR pathway²³. The AKT/mTOR pathway typically promotes muscle growth, however when stimuli such as inactivity, disease, injury and aging disregulates the pathway through AKT dephosphorylation, it prevents AKT from phosphorylating the pro-degradatory forkhead transcription factor FoxO1^{3,20,23-25,31-34}. FoxO1 which consists of four domains: a forkhead DNA-binding domain, a nuclear localization signal, a nuclear export sequence, and a C-terminal transactivation domain^{3,12,27} is regulated by AKT. AKT creates sites for 14-3-3 binding on FoxO1 proteins which in turn promotes further phosphorylation by casein kinase-1 and tyrosine kinase-1A^{3,4,12,27,32}. Collectively the formation of this complex inhibits FoxO1's function and keeps it sequestered in the cytosol^{3,4,12,23,27,32,37}. However when FoxO1 is dephosphorylated it becomes active and

translocates to the nucleus where it promotes upregulation of various transcription factors²³. Studies have shown that in a variety of tissues FoxO1 has been found to regulate atrophy through both proteolytic and apoptotic pathways^{1,12,15,20,21,24,27,37}.

FoxO1 most notoriously regulates protein ubiquitination by upregulating the expression of genes Muscle Atrophy F-box (Atrogin-1/MAFbx) and Muscle Ring Finger1 (Murf-1)^{22,29,34,35}. These genes when overexpressed, promote the formation of the ubiquitin complex composed of ubiquitin and E3 ligases^{29,35,39}. The ubiquitin complex in turn selectively targets specific proteins for degradation. Once the proteins of interest are tagged with ubiquitin they are rapidly degraded by the 26S proteasome^{22,29,35,39}.

Another form of atrophy seen within skeletal muscle is caused by apoptosis or programmed cell death. This intracellular death pathway kills cells in a controlled fashion through compartmentalization of cellular components which are then phagocytosed^{28,29}. The process is characterized by DNA fragmentation, loss of nuclei, and mitochondrial disruption and remodeling^{7,29,41,42}. Furthermore, it can be regulated through two different routes which include the mitochondria (intrinsic route) and the activation of death receptors (extrinsic pathway)^{13,38,42}. Several studies on muscle atrophy have found that mitochondrial disruption is a prominent characteristic seen in the muscle wasting process; therefore, it appears that the primary route of apoptosis within skeletal muscle is intrinsic^{6,8-10,39}. The intrinsic apoptotic pathway is controlled primarily by the ratio of pro and anti-apoptotic proteins within the mitochondria^{6,38,42}. Pro-apoptotic genes Bim and BNip3 act on anti-apoptotic Bcl-2, inhibiting its function^{5,6,14,15,19,28,43}. Once Bcl-2 is inhibited, cytochrome c is released into the cytoplasm and binds with Apaf-1 and Pro-caspase 9 to form an apoptosome^{7,10,13,38,42}. The formation of this complex in turn

activates caspase-9 which will in turn activate the rest of the effector caspases^{7,10,11,13,38,42}.

Within various tissue types excluding skeletal muscle FoxO1 and related isoforms have been found to promote expression of apoptotic genes Bim and BNip3 through both DNA dependent and independent mechanisms^{1,12,32}.

Our previous data has shown indications that FoxO1 activation promotes muscle atrophy evidenced through a decrease in protein content accompanied by signs of apoptosis through DNA fragmentation. Signs of this relation suggest further investigation into the role of FoxO1 in regulating apoptosis within skeletal muscle atrophy by investigating its role in promoting expression of apoptotic genes such as Bim and BNip3 in addition to genes Murf-1 and Atrogin-1/Mafbx within the ubiquitin-proteasome pathway. Therefore, the purpose of this study is to examine the link between FoxO1 expression, protein degradation and apoptosis during skeletal muscle atrophy. Plus, we further hope to elucidate the contribution of promoter binding to FoxO1 mediated skeletal muscle atrophy. We have hypothesized that FoxO1 expression in skeletal muscle cells would result in atrophy associated with protein ubiquitination and apoptosis. Furthermore, FoxO1-mediated muscle atrophy and associated gene expression would rely on FoxO1-DNA binding.

Chapter Two

Literature Review

2.1 Mechanisms of Skeletal Muscle Atrophy

The primary functions of skeletal muscle are to provide power and strength for locomotion and posture in addition to acting as a store for body proteins and thermoregulation. In order for skeletal muscle to function at optimal efficiency it is imperative that skeletal muscle proteins maintain homeostasis between synthesis and degradation. When the homeostatic balance is off amongst the proteins, protein degradation occurs. This loss of skeletal muscle mass yields deterioration in the performance of activities of daily living in addition to dampened metabolic function due to the change in physiological demands caused by the muscle loss^{9,35,39,40}. Muscle atrophy can be triggered by several mechanisms, some of which include: neuromuscular disease, chronic disease, immobilization, injury and aging^{8,9,20,22,23,32,34-36,40,44}. In the paragraph to follow I will divulge some of the details as to how these various mechanisms trigger muscle atrophy.

At a glance, the mechanisms that trigger muscle atrophy appear to be diverse and have little in common; however the exact opposite is true. Many of the mechanisms in one form or other trigger similar molecular pathways in order to induce skeletal muscle atrophy^{6,8,9,12,14,20,22,30,35,36,39}. For example, in neuromuscular diseases, chronic diseases and aging there is significant accumulation of mitochondrial DNA mutations^{8,9,15,22,30}. The accumulation of mitochondrial DNA mutations compromises the integrity of the cell triggering the apoptotic pathway^{8,9,14,22,30}. Furthermore, in heart disease, various injuries and in aging again; ATP production can be inhibited under hypoxic conditions which triggers both proteolytic and apoptotic degradation^{6,8,9,17,20,30,35,39}. Collectively as you can see, any compromise in mitochondrial integrity, oxygen or ATP supply will trigger protein degradation and apoptosis within skeletal muscle cells. Due to these observations much research has been done in understanding the specifics of these catabolic pathways and many have found that AKT is a pivotal point in atrophy signaling pathways^{3,4,15,20,23,24,27,31-33,37}. On a phenotypic level the activation of these pathways results in decreased muscle cross sectional area, mitochondrial dysfunction, loss of myonuclei and of course protein degradation^{8,17,25,30,35,39,44}. Since health care costs due to muscle atrophy run up to 18.5 billion (US) per year, it is imperative that advances in understanding atrophy using pharmacological and genetic manipulation in cells and rodents continues to take place in order to find a treatment or cure for muscle atrophy^{8,9,23-25,34,35}.

2.2 Protein Ubiquitination

Within skeletal muscle half of muscle protein is made of up myofibril protein, this fraction of protein is lost at a faster rate than other muscle proteins in skeletal muscle

atrophy^{9,20,22,39}. A majority of the proteolysis in muscle appears to be due to the activation of the ubiquitin-proteasome pathway^{20,22,35,39}. The ubiquitin-proteasome pathway is a major extralysosomal pathway that is responsible for promoting the intracellular degradation of proteins^{22,29}. In fact, several models of muscle atrophy; including: starvation, uremia, denervation, sepsis, and diabetes mellitus have activated the ubiquitin-proteasome pathway^{20,35}.

Although there is substantial variability in the physiological and pathophysiological stimuli for muscle atrophy, it appears that there are striking similarities in transcriptional adaptations of genes coding for the ubiquitin-proteasome pathway²². The ubiquitin-proteasome pathway is composed of a ubiquitin conjugating system and 26S proteasome²⁹. This system is comprised of several classes of enzymes; ubiquitin activating enzymes (E1) which modify ubiquitin so that it is in a reactive site, ubiquitin conjugating enzyme (E2) which catalyzes the attachment of the ubiquitin protein, and ubiquitin ligases (E3) which functions to recognize the substrate protein and aid E2 enzymes in the attachment to the substrate protein^{20,29}. Together this complex tags specific proteins for degradation by the 26S proteasome^{29,35}.

In 2001, following a screening for genes upregulated in different models of rodent muscle atrophy, genes Atrogin-1/MAFbx and Murf-1 were discovered^{2,16}. Since their discovery, several studies including those looking at cardiomyopathies have found that Atrogin-1/MAFbx and Murf-1 are the key E3 ligases utilized in muscle atrophy thorough protein ubiquitination^{24,34,35}. In fact, in Murf-1 knockout mice subjected to muscle denervation, there was a 36% decrease in the amount of muscle atrophy at 14 days. Likewise in Atrogin-1/MAFbx knockout mice 56% of muscle was spared from

atrophy^{20,34}. Other E3 ligases such as Nedd4 and Mdm2 have also been investigated for their contribution to muscle atrophy however little has yet to be elucidated, most likely their key contributions are towards myotube growth²⁰. With resistance training the effects of Atrogin-1/MAFbx and Murf-1 have found differences in their degrees of expression with Murf-1 expression continuing to increase while Atrogin-1/MAFbx is down regulated²⁵. The difference in expression indicates a divergence in their regulation abilities and that they may have different functions in protein degradation^{20,25}.

2.3 Apoptosis

Apoptosis or programmed cell death is a distinct intrinsic cell death program that occurs in various physiological and pathological situations^{7,10,14,18,28,29,42}. It is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation, membrane blebbing, and mitochondrial disruption and remodeling^{7,29,42}. This degradative pathway allows for the death and removal of a single cell without disturbing the surrounding tissue^{9,13} and is therefore, essential for the development and maintenance of tissue homeostasis in highly proliferative cells such as those found in the intestine, liver, white blood cells and stem cells. Although apoptosis is seen extensively in highly proliferative cells, it is still somewhat controversial as to whether or not apoptosis occurs in postmitotic tissue such as the brain, heart and skeletal muscle^{1,6,8-10,17,18,37}.

Apoptosis as a whole occurs through the activation of cysteine proteases (caspases) that dismantle cells by degrading essential proteins^{9,10,13,38,39,42}. The process consists of two interrelated pathways: an extrinsic (death receptor pathway) and an

intrinsic (mitochondrial pathway); activation of which depends on the cellular stresses placed on the cell. The extrinsic pathway is triggered by ligands binding to the plasma membrane death receptors. This union activates initiator caspase 8; depending on the cell type, it then activates effector caspase 3 or it engages the intrinsic pathway. The intrinsic pathway is controlled by ratio of pro and anti-apoptotic Bcl-2 proteins. In addition to varying ratios of pro and anti-apoptotic Bcl-2 proteins some types of outside stimulus can alter protein interactions on the mitochondrial surface independently^{9,10,13,38,39,42}.

Any alterations in mitochondrial integrity cause the release of cytochrome-c into the cytosol. Cytochrome-c in turn interacts with Apaf-1, pro-caspase 9, and dATP to form an apoptosome. The apoptosome complex then activates caspase 9 which then triggers the activation of effector caspase 3^{8,9,13,14,30,38,39,42,44}. Caspases as a whole are synthesized as inactive zymogens and are activated by proteolytic cleavage. Initiator caspases transduce various signals into protease activity and are directly linked to death inducing signaling complexes; whereas, effector caspases cleave various cytoplasmic or nuclear substrates^{7-9,11}.

Many of the key components that initiate and execute apoptosis have been found to be conserved across species⁴¹. However, detection of apoptosis in vivo is difficult because it is asynchronous and the half life of apoptotic cells in tissue is short⁴². Thus far, through extensive research apoptosis has been found to occur within the cardiovascular system, the neurological system and skeletal muscles as a result of a number of stimuli including: hypoxia, denervation, immobilization and aging^{1,6,8-10,14,17,30,37,39}.

Apoptosis plays an important role within the cardiovascular system not only in the process of homeostasis and development but also in the pathogenesis of certain diseases^{6,18,37}. It is implicated in the death of myocytes in animal models of myocardial ischemia, and in humans with acute myocardial infarction and congestive heart failure¹⁸. Cell death induced by ischemic stress of cardiomyocytes occurs through the mitochondrial pathway which is partially regulated by Bcl-2 family proteins^{6,18,37}.

Pro-apoptotic protein BNip3 (Bcl-2 and nineteen-kilodalton interacting protein-3) is strongly activated by hypoxia such as that seen with cardiac damage⁶. In fact through subtractive hybridization studies, BNip3 has been found to be the most prominent hypoxia responsive gene^{5,6}. BNip3 functions through binding to and inhibiting anti-apoptotic Bcl-2 which allows cytochrome-c to be released from the mitochondria leading to degradation⁵. In addition to cardio myocytes BNip3 has also been found to induce apoptosis in fibroblasts and neuroblastoma cells through the interaction of transcription factors such as PLAGL2 (pleomorphic adenomas gene-like 2), E2F1, HIF-1• (hypoxia-inducible factor) and FoxO3^{5,6}. Through interaction with FoxO3, BNip3 is capable of inducing another form of cell degradation called autophagy⁵. BNip3's ability to induce autophagy is dependent upon binding with Bcl-2 members Bax and Bak following which mitochondrial integrity is disrupted and the autophagic pathway is further activated⁵. Therefore, it appears that BNip3 can interact with both apoptotic and autophagic transcription factors⁵. Furthermore, in studies utilizing neonatal rat cardiac myocytes exposed to hypoxia and acidotic conditions BNip3 caused cell death resembling apoptosis that was independent of caspase activation suggesting that BNip3 induces both apoptotic and non-apoptotic cell death depending on the stimulation and/or cell type^{5,6}.

Programmed cell death accounts for the death of approximately half of all neurons generated during embryogenesis. With embryogenesis, apoptosis is essential for correct innervations of target tissues and formation of neuronal networks during neural development¹⁴. Within sympathetic neurons deprivation of NGF activates the intrinsic apoptotic pathway. The activation of this pathway within neurons specifically activates pro-apoptotic protein Bim¹⁴. Bim functions very similar to that of BNip3 in inhibiting anti-apoptotic Bcl-2. However, Bim consists of three isoforms that produce varying degrees of cytotoxicity against Bcl-2²⁸. Furthermore, research thus far has shown Bim to be stronger than BNip3 in stimulating mitochondrial mediated apoptosis⁵. Neural cells are not the only tissue Bim regulates apoptosis in; many other cell types including several cancer cell lines have shown increased activation of Bim. Likewise, many FoxO family proteins are found throughout tissues in the body and as of recent, attention has begun to be paid to the induction of apoptosis through pathways involving FoxO/Bim interaction.^{14,19,28}

Apoptosis has been documented to occur in muscle dystrophy, chronic heart failure, skeletal muscle denervation, muscle unweighting and during acute exercise^{8-10,17,18,30,37,39,44}. However, relatively few studies have been published looking at the role of apoptosis in loss of muscle mass and function in aging muscle^{8,9}. In most cell types removal of the nuclei equates into apoptosis of the entire cell, unlike other cells in the body, skeletal muscle is multinucleated so apoptosis of a nucleus may not cause apoptosis of the entire cell^{8-10,39}. The process by which nuclei are removed from muscle fibers resembles apoptosis, however since destruction of the entire cell does not occur, the process within skeletal muscle is termed “apoptotic nuclear death”^{10,17}.

It has been postulated that the loss of cells in post-mitotic tissue such as skeletal muscle via apoptosis activation contributes to the age-related decline in function^{8,9,30,44}. Current research has noted that aged skeletal muscle is characterized by increased mitochondrial dysfunction which correlates with the fact that mitochondrial dysfunction is a potent mechanism of apoptotic induction via the internal pathway^{8,9,30,44}. Within the mitochondria several key death factors reside between the inner and outer membrane¹¹. In addition to Bim and BNip3, caspase independent AIF (apoptosis inducing factor) and endonuclease G (endo-G) are located within the mitochondria and upon disruption, can cause large scale DNA fragmentation and apoptosis by translocating to the nucleus^{11,41}. One study has found that endo-G co-localization with the nuclei increased in muscles atrophied by hind limb suspension and age and concentrations further increased with aged rats undergoing disuse induced atrophy⁹. Furthermore, it has also been found that endo-G translocation is very specific for myonuclei and not involved in apoptosis of interstitial cells⁹. Even with evidence of endonuclease G translocating to the myonuclei in apoptosis there is still much to be discovered behind the mechanisms involved in myonuclear loss in atrophy^{10,17}. AIF is a flavin-adenine dinucleotide (FAD)-binding oxidoreductase, however neither its FAD-binding activity nor its oxidoreductase activity is required for its apoptogenic activity⁴¹. Therefore, it has been suggested that AIF's action is that which is utilized to remove unnecessary or unused muscle tissue in an effort to conserve the proteins for other bodily needs in aging and that AIF is the unknown mechanism responsible for myonuclei removal^{11,41}.

2.4 FoxO1

Transcription factors are modular proteins with distinct functions contained within defined domains, such as DNA-binding and transactivation of transcription³. The forkhead family of transcription factors are characterized by a type of DNA-binding domain known as the forkhead box (FOX)^{3,4,27,31}. The forkhead family is also called winged helix transcription factors because of the crystal structure of the Fox. Furthermore, the Fox name was given to the protein after its' founding gene member was discovered in *Drosophila*^{3,4,27,31}. Genes encoding forkheads are present throughout the animal kingdom as well as in yeast and fungi³¹. In humans specifically, over 100 genes encoding forkhead proteins are present in the genome; therefore, due to the vast number, the forkhead proteins are classified into subgroups based on sequence similarity³¹.

The O subfamily of forkhead (Fox) proteins regulates hormonal, nutrient, and stress responses to promote cell survival and metabolism^{3,4,12,20,27,31,32}. Of the numerous subgroups they are the most divergent of the Fox family due to a unique five amino-acid insertion found prior to the helix H3 within the forkhead domain. This domain has been found to be directly involved in sequence specific interaction with DNA-binding sites. Specifically, FoxO factors including FoxO1, FoxO3a, FoxO4 and FoxO6 share DNA-binding specificity to a core consensus site called the forkhead-responsive element and regulate the transcription of genes involved in several cellular processes including: cell cycle arrest, apoptosis, DNA repair in response to oxidative stress, differentiation, glucose metabolism and aging^{3,12,27}.

Prototypic regulation of FoxO function is characterized by activation of the insulin-signaling pathway in mammals, FoxO proteins are targets of growth-factor-stimulated AKT phosphorylation^{3,4,12,14,15,23,24,27,31-34,37,43}. Insulin/IGF-1 stimulates AKT activation leading to phosphorylation of FoxO proteins at threonine 24, serine 256 and serine 319 on FoxO1^{4,27,33}. In greater detail, the phosphorylation of serine 256 inhibits transactivation, while phosphorylation of threonine 24 induces interaction with 14-3-3 proteins sequestering FoxO1 to the cytosol³³. As far as phosphorylation of serine 319, at this point little is known about its role, although it is believed to contribute to nuclear exclusion³³. Collectively, the functional consequence of AKT mediated FoxO phosphorylation is a nuclear-cytoplasmic shuttling of FoxO, thus inhibiting transcription of FoxO target genes^{3,4,12,14,15,23,24,27,31-34,37,43}.

In numerous studies within cell types including: neurons, skeletal and smooth muscle, hematopoietic cells and fibroblasts; FoxO proteins have been found to transcribe genes in both the ubiquitin-proteasome and apoptotic pathways^{1,12,15,20,21,24,27,37}. For example, *in vitro* and *in vivo* rodent studies have consistently shown that under catabolic conditions Atrogin-1/MAFbx and Murf-1 genes are regulated by pathways in which FoxO transcription factors are activated^{12,15,20,23-25,34}. Furthermore, in skeletal muscle and cardiomyocytes FoxO isoforms have also been found to regulate Atrogin-1/MAFbx through the ubiquitin-proteasome pathway^{24,34}. In regards to apoptosis, cell stresses that suppress AKT signaling and activate FoxO1 will induce Fas ligand (FasL) expression on the vascular smooth muscle cell surface via a caspase pathway³⁷. Fas is a type I membrane protein that transmits a suicide signal to the cell. The clustering of Fas following the binding of FasL or anti-fas antibodies leads to caspase 8-dependent cell

death³⁷. As stated previously, in addition to FasL, FoxO1 proteins can also regulate other pro-apoptotic genes including Bim and BNip3 in both DNA-dependent and -independent mechanisms^{1,12,32}.

Due to the mounting evidence that FoxO1 regulates genes in both proteolytic and apoptotic pathways in a variety of tissues; the purpose of this study is to examine the link between FoxO1 expression, protein degradation and apoptosis during skeletal muscle atrophy. Furthermore, we hope to elucidate the contribution of promoter binding to FoxO1 mediated skeletal muscle atrophy. We hypothesized that FoxO1 expression in skeletal muscle cells would result in atrophy associated with protein ubiquitination and apoptosis. In addition, FoxO1 mediated muscle atrophy and associated gene expression would rely on FoxO1-DNA-binding.

Chapter Three

Materials and Methods

3.1 Retroviral Transfections

FoxO1-estrogen receptor (FoxO1-ER) fusion proteins that are activated by treatment of 4 OH-T were stably expressed in C2C12 skeletal myoblasts using a pBABE retroviral system. Briefly, cells were seeded in flat-bottom culture plates and grown in DMEM supplemented with 10% FBS (culture medium). At ~50% confluence, cells were placed in culture medium supplemented with polybrene (10 µg/ml) and transfected overnight with one of four retroviral constructs. Positively transfected cells were selected by incubating myoblasts in culture medium supplemented with puromycin (Specialty Media, Temecula, CA; 3.5 µg/ml) for 24 hours. The two retroviral constructs included FoxO1^{AAA-ER} and FoxO1^{AAA/Arg215-ER}. AAA corresponds to the alanine mutations of the here putative FoxO1 Akt phosphorylation sites, Thr²⁴, Ser²⁵⁶, and Ser³¹⁹. AAA/Arg215 contains an additional mutation, H215R, which is in the DNA binding domain and disrupts promoter binding. Non-transfected C2C12 cells served as the control.

3.2 Skeletal Myotube Culture

Mouse C2C12 cells were maintained in low glucose Dulbecco's modified Eagle's Medium (DMEM; Invitrogen Inc., Carlsbad, CA) supplemented with 10% FBS (culture medium). At 80-90% confluence, cells were switched to DMEM supplemented with 2% horse serum (differentiation medium) for 4 days. On the 4th day, myotubes were treated with differentiation medium supplemented with either vehicle (ethanol: 0.1% final concentration) or 4-hydroxy tamoxifen (4 OH-T; 500 nM; Sigma-Aldrich, St. Louis, MO) for up to 48 hours.

3.3 Protein Quantification

For protein quantification, C2C12 myotubes were collected in a buffer containing 10 mM MgCl₂, 10 mM KH₂PO₄, 1 mM EDTA, 5 mM EGTA, 50 mM •-glycerolphosphate, 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptin, 10 mM aprotinin, and 10 mM Na₃VO₄. Total protein concentration was assayed with a detergent-compatible protein assay kit (DC Protein Assay, Bio-Rad, Hercules, CA) and quantified by spectrophotometry using a microtiter plate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA).

3.4 Gene Expression Analysis

Total RNA was isolated using an Rneasy Mini column (Qiagen, Valencia, CA), purified by DNase digestion (Turbo DNase; Ambion, Foster City, CA), and stored at -80° C until further processing. Total RNA was separated by agarose gel electrophoresis and ribosomal bands examined to ensure sample quality. cDNA synthesis using 10 ng of total

RNA and subsequent PCR amplification was performed using a one-step qRT-PCR kit (Superscript III, Invitrogen, Inc., Carlsbad, CA) and gene specific Taqman[®] primers and probes (Applied Biosystems, Foster City, CA) designed against Atrogin-1/MAFbx (Mm00499518_m1), Murf-1 (Mm01188690_m1), Bim (Mm00437795_m1), BNip3 (Mm00833810_g1) and GAPDH (Mm99999915_g1). The qRT-PCR reactions were carried out in triplicate using a 7500 Real-Time Detection System (Applied Biosystems). Analyses were performed to verify the dynamic range and confirm consistency amongst the amplification efficiencies of the various target genes analyzed. Data was expressed via the comparative C_t method, in which • C_t values were calculated for all samples as follow: C_t_{target gene} – C_t_{housekeeping gene}, where the target genes were Atrogin-1/MAFbx, Murf-1, Bim, and BNip3 and the housekeeping gene was GAPDH. Relative changes in gene expression were then calculated for each target gene via the 2^{–• C_t} method, in which • C_t values determined for each of the experimental samples (4 OH-T treated) were subtracted from the • C_t value from the calibrator sample (vehicle treated). The stability of the GAPDH housekeeping gene was confirmed through C_t analysis, which revealed that the range for average C_t values for the various conditions were within a ½ C_t.

3.5 Statistical Analyses

A two-way factorial ANOVA (p • 0.05) was used to determine differences between conditions for all dependent variables assessed. Neuman-Keuls post-hoc analyses (p • 0.05) were used to locate differences between means if a significant main effect and/or interaction was found.

Chapter Four

Results

Impaired DNA binding results in differential gene expression conferred through FoxO1 activation.

Treatment with 4 OH-T did not alter the expression of target genes in non-transfected C2C12 cells or FoxO1^{EV-ER} cells following vehicle or 4 OH-T treatment, so expression data for all gene targets were compared to the non-transfected C2C12 cells. As shown in Fig. 1, Atrogin-1/MAFbx gene expression was increased ~27 fold following 24 hour treatment with 4 OH-T in FoxO1^{AAA-ER} cells. In contrast, Atrogin-1/MAFbx gene expression was unchanged in the non-transfected cells and the FoxO1^{AAA/Arg215-ER} cells following exposure to 4 OH-T (Fig. 4.1).

Murf-1 gene expression was elevated in a similar manner following 4 OH-T treatment for the FoxO1^{AAA-ER} (~2 fold) and FoxO1^{AAA/Arg215-ER} cells (~2.2 fold), which were both greater than gene expression measured in the non-transfected condition (Fig.

4.2), indicating that FoxO1 can regulated the expression of Murf-1 through a mechanism that is independent of DNA binding.

Gene expression of Bim was significantly elevated in both the FoxO1^{AAA-ER} (~3.5 fold) and the FoxO1^{AAA/Arg215-ER} (~2 fold) cells, both of which were greater than the non-transfected condition (Fig. 4.3). Interestingly, the increase in Bim gene expression observed in the FoxO1^{AAA-ER} cells was significantly greater than the increased expression observed in the FoxO1^{AAA/Arg215-ER} cells, indicating that FoxO1 regulates the expression of Bim through both DNA binding-dependent and independent mechanisms.

BNip3 gene expression was unchanged in the FoxO1^{AAA-ER} cells following administration of 4 OH-T (Fig. 4.4). Interestingly, FoxO1^{AAA/Arg215-ER} cells exhibited a significant increase of BNip3 gene expression following 4 OH-T treatment (~2.2 fold), indicating that an alternative apoptotic gene expression profile results following FoxO1 activation in the absence of DNA-binding.

Figure 4.1: Atrogin-1/MAFbx relative gene expression for non-transfected, FoxO1^{AAA-ER} (AAA), and FoxO1^{AAA/Arg215-ER} (215) myotubes following treatment with either vehicle (VEH) or 4 OH-T for 24 hours. Brackets denote location of statistical significance (* = p < 0.05). n = 4-6 per condition.

Figure 4.2: Murf-1 relative gene expression for non-transfected, FoxO1^{AAA-ER} (AAA), and FoxO1^{AAA/Arg215-ER} (215) myotubes following treatment with either vehicle (VEH) or 4 OH-T for 24 hours. Brackets denote location of statistical significance (* = $p < 0.05$). $n = 4-6$ per condition.

Figure 4.3: Bim relative gene expression for non-transfected, FoxO1^{AAA-ER} (AAA), and FoxO1^{AAA/Arg215-ER} (215) myotubes following treatment with either vehicle (VEH) or 4 OH-T for 24 hours. Brackets denote location of statistical significance (* = $p < 0.05$). $n = 4-6$ per condition.

Figure 4.4: BNip3 relative gene expression for non-transfected, FoxO1^{AAA-ER} (AAA), and FoxO1^{AAA/Arg215-ER} (215) myotubes following treatment with either vehicle (VEH) or 4 OH-T for 24 hours. Brackets denote location of statistical significance (* = $p < 0.05$). $n = 4-6$ per condition.

Chapter Five

Discussion

The prominent findings from this study reveal that muscle atrophy consequential of FoxO1 protein activation culminates from various mechanisms, including increased activity of protein ubiquitination and apoptotic machinery. While the ability of FoxO1 to induce muscle atrophy does not appear to be dependent on DNA binding; its effects on protein ubiquitination and apoptosis appear to be caused by a direct interaction of FoxO1 and promoter binding sites.

Great evidence concerning FoxO1 expression to skeletal muscle atrophy through the upregulation of genes associated with muscle protein degradation, such as Atrogin-1/MAFbx and Murf-1, has been shown in many in vitro and in vivo studies^{12,15,20,23-25,34}. Our findings indicate that in relationship to the hearty increase in Atrogin-1/MAFbx gene expression, Murf-1 activation was relatively blunted. Our findings also suggest that the ability of FoxO1 to regulate Atrogin-1/MAFbx expression is highly dependent upon DNA binding, while Murf-1 gene expression did not appear to be highly contingent upon FoxO1-DNA binding. Although it can not be distinguished from our data, the differential

response in expression among these ubiquitous proteins may be due to dissimilar roles in regulating muscle homeostasis. For example, in a study done by Mascher et al. looking at the effects of resistance training on mRNA expression of Atrogin-1/MAFbx and Murf-1, they found that following repeated exercise sessions Murf-1 gene expression increased while Atrogin-1/MAFbx expression remained the same as pre exercise levels.

Furthermore, in a review by Aaron P. Russel on the molecular regulation of skeletal muscle mass, he provides evidence that the magnitude of Murf-1 and Atrogin-1/MAFbx gene expression varies due to type of organism (rodent vs. human) and the mechanism (ie. disease, denervation, activity level) impacting skeletal muscle integrity.

Together with these changes in proteasome-related genes, we saw distinct findings in terms of protein ubiquitination subsequent activation of FoxO1 in the various cell lines. Particularly, total protein ubiquitination appeared to be elevated in the FoxO1^{AAA-ER} cell line following 4 OH-T treatment, while FoxO1^{AAA/Arg215-ER} cells did not. This evidence provides further support that Atrogin-1/MAFbx is highly dependent upon FoxO1 DNA binding and additionally that Atrogin-1/MAFbx and Murf-1 may play slightly different roles in the induction of skeletal muscle atrophy.

However, given the strong ability of Atrogin-1/MAFbx to induce protein degradation, it is probable that the suppressed generation of Atrogin-1/MAFbx observed in the FoxO1-DNA binding deficient cells may account for limited protein ubiquitination following FoxO1 activation. Interestingly, bearing in mind that muscle atrophy and total protein loss was still seen in the DNA-binding deficient condition in spite of a blunting in Atrogin-1/MAFbx expression and total protein ubiquitination it is therefore probable that

mechanisms beyond proteasomal-mediated degradation play a role in muscle atrophy following FoxO1 activation.

One of the more distinguished cellular events coupled with the re-modeling of atrophied skeletal muscle fibers is the loss of myonuclei, probably triggered through the commencement of the pro-apoptotic pathways. Even though the role of FoxO1 on apoptosis within skeletal muscle has not been fully elucidated, the activation of FoxO1 and related isoforms has been extensively implicated in initiating cellular events yielding apoptosis and/or cell cycle arrest in a variety of proliferative cells^{1,12,15,20,21,24,27,37}.

Apoptotic signaling induced via FoxO1 expression appeared reliant upon DNA binding, as pro-apoptotic Bim expression was less prevalent in FoxO1^{AAA/Arg215-ER} compared to expression within the FoxO1^{AAA-ER} condition. Bim gene expression witnessed in the FoxO1^{AAA/Arg215-ER} cell line was likely due to trace DNA binding, at the cell line results in suppression rather than complete elimination of FoxO1-DNA binding. It is possible that abrogated FoxO1-DNA binding caused a delay rather than a complete loss in apoptotic signal transduction. However due to difficulties in maintaining proper muscle cell integrity with prolonged exposure to 4 OH-T we were unable to achieve reliable data beyond 24 hours for histochemical measurements. Therefore, it looks as if the differences in apoptotic signaling witnessed between the FoxO1^{AAA-ER} and FoxO1^{AAA/Arg215-ER} cell lines were the result of differences in DNA binding capability.

To our surprise, BNip3 established a different response, in that gene expression remained unchanged in FoxO1^{AAA-ER} cells, yet was significantly elevated in FoxO1^{AAA/Arg215-ER} cells. During apoptosis, BNip3 seems to function as an allosteric

protein, requiring union with either Bax or Bak to make cell death possible⁵. When apoptosis is not activated, which appears to be the case in FoxO1^{AAA/Arg215-ER} cells can disrupt mitochondrial integrity by possibly activating alternate forms of cell death such as autophagy^{5,6,13}. Known that the FoxO family can interact with a wide array of distinct transcription factors^{1,12,15,20,23-25, 32, 34} it is possible that in the absence of direct promoter binding, FoxO1 may be controlling genes through protein-protein interactions and activating cell death pathways separate from that viewed in DNA-binding capable cell lines.

The conclusions drawn from this study supply further insight into the multifaceted role of FoxO1 within the biological regulations of skeletal muscle. In particular, in remodeling of atrophied skeletal muscle generated through FoxO1 activation, may rely upon both DNA binding dependent and independent mechanisms. Although, in vitro work is no where near the complexity of that of an integral physiological system, it is feasible that the increases in FoxO1 expression may be a powerful driving force for breakdown and removal of muscle proteins and nuclei through the activation of apoptotic machinery associated with conditions such as neuromuscular disease, chronic disease, immobilization, injury and aging. In light of FoxO1's intricate ability to regulate cellular function, thorough contemplation should be given in the development of pharmaceutical, gene therapy and rehabilitation approaches in endeavors to hinder FoxO1-induced muscle atrophy.

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