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The impact of FoxO1 on skeletal muscle protein synthesis

Rachael Ann Potter
University of Toledo

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The Impact of FoxO1 on Skeletal Muscle Protein Synthesis

by

Rachael A. Potter

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in

Exercise Science

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Dr. Thomas J. McLoughlin, Committee Chair

_____

Dr. Francis X. Pizza, Committee Member

_____

Dr. Joseph Marino, Committee Member

_____

Dr. Abraham Lee, Committee Member

_____

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

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An Abstract of

The Impact of FoxO1 on Skeletal Muscle Protein Synthesis

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The regulation of skeletal muscle is dependent upon the balance between protein synthesis and protein degradation. The FoxO1 transcription factor engages in an important role in regulating skeletal muscle hypertrophy through upregulation of atrophy-related genes (i.e. MAFbx/atrogin-1). Studies have shown that FoxO1 regulates skeletal muscle atrophy through both apoptotic and proteolytic pathways in various tissues.[3,8,37,80] However, the interaction between FoxO1 overexpression and protein synthesis is unknown. The hypotheses tested include: i) the overexpression of FoxO1 in vitro suppresses skeletal muscle protein synthesis and ii) suppression of skeletal muscle protein synthesis is due to suppression of ribosome biogenesis. An in vitro model was used in which FoxO1 estrogen receptor fusion proteins were transfected into skeletal muscle myoblasts and grown into myotubes. The differentiated myotubes were treated with 4-hydroxytamoxifen (4-OHT) to activate the FoxO1 estrogen receptor fusion proteins and with insulin as a hypertrophic stimulus for 30 minutes, 60 minutes, and 120 minutes. The cells were treated with [^{3}H] phenylalanine to measure total protein

synthesis upon FoxO1 overexpression. In addition, expression of key anabolic molecules including Akt, $p70^{s6k}$, ribosomal protein S6, and Cyclin D1 activity was assessed via western blot. Our findings show that i) FoxO1 overexpression significantly suppresses protein synthesis in differentiated myotubes, ii) despite treatment with insulin; FoxO1 overexpression blunts protein synthesis compared to control myotubes, iii) FoxO1 overexpression suppresses activation of Akt and ribosomal protein S6. In addition, insulin treatment did not elevate phosphorylation of ribosomal protein S6 upon FoxO1 overexpression. In this study, the major finding is that FoxO1 overexpression suppresses protein synthesis prior to any phenotypic loss of protein content in a ribosomal protein S6 dependent manner. In addition, total protein synthesis is suppressed despite treatment with insulin. Therefore, decreased skeletal muscle size is attributed to both increased protein degradation and suppression of protein synthesis. It can be speculated that the suppression of protein synthesis is due, at least in part, to alterations in ribosomal biogenesis. Future direction will focus on ribosomal biogenesis to elucidate the mechanism to which FoxO1 suppresses protein synthesis.

This is dedicated to those who have had nothing but endless support for me through this long journey. To my husband, Joe, who has always believed in me. To my parents, who have encouraged me and supported me through this process. And to Baby Potter, may you always be motivated and never give up on your dreams!

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Chapter 1

Introduction

Background and Significance

Muscle wasting is concomitant with many chronic metabolic diseases and affects millions of people worldwide. Obesity and associated disorders are quickly on the rise and it is estimated that over 500 million people worldwide are considered obese or overweight.[91] To make matters worse, obesity is highly correlated with the development of metabolic syndrome, type 2 diabetes, and cardiovascular disease .[69] Maintenance of healthy muscle is imperative to fend off metabolic disease and maintain normal muscle function.[2] The molecular mechanisms surrounding muscle atrophy and hypertrophy are essential in order to develop therapies to combat metabolic disease. Skeletal muscle mass can change rapidly in response to various stimuli, including nutrients, neural activity, cytokines, growth factors, hormones and mechanical load.[36,44,111] These changes evoke alterations in protein synthesis and protein degradation. Thus, mechanisms that activate protein synthesis machinery leads to skeletal muscle growth and mechanisms that activate protein degradation leads to skeletal muscle atrophy.[40,50] The literature provides a strong base of research supporting the role of Akt and $p70^{s6k}$ as markers of anabolism.[11] In addition, the literature corroborates the role of phosphorylation of Ribosomal Protein S6 (rpS6) in the initiation of mRNA translation and protein synthesis.[32,59,129]

The FoxO1 transcription factor appears to play an important role in regulating skeletal muscle hypertrophy through upregulation of atrophy-related genes (i.e. MAFbx/atrogin-1). Studies have shown that FoxO1 regulates skeletal muscle atrophy through both apoptotic and proteolytic pathways in various tissues.[3,8,37,80] However, the interaction between FoxO1 overexpression and protein synthesis is unknown. Recently, using an in vivo model, our laboratory has demonstrated that FoxO1 skeletal muscle specific overexpression results in blunting of muscle hypertrophy after chronic overload of the muscle. This blunting of muscle hypertrophy occurs concomitantly with hyperphosphorylation of Akt. In addition, there were no alterations in mTOR or $p70^{s6k}$ activity in FoxO1 overexpressing mice, upon chronic overload.[96] Therefore, blunting of muscle hypertrophy in FoxO1 overexpressing mice occurs despite intact Akt signaling. Previous literature identifies the controversial nature of signaling through Akt, mTOR, and $p70^{s6k}$ in muscle mass maintenance, thus it is important to digress from the evaluation of this "canonical" Akt/mTOR pathway.[26,35,51,75,85,96] In fact, it is imperative to evaluate the interaction between FoxO1 and protein synthesis machinery in order to better understand the regulation of skeletal muscle mass. The goal of this project, through the use of an inducible FoxO1 overexpressing cell culture model, is i) to assess the impact of FoxO1 overexpression on protein synthesis, and ii) to determine if protein synthesis suppression occurs through inhibition of Akt, $p70^{s6k}$, and rpS6 activation. The *long-term goal* of this project is to elucidate the molecular mechanisms responsible for muscle wasting diseases in order to identify effective treatment options. The *objective* of this project is to use total protein synthesis measurements and interactions

with anabolic signaling molecules during an acute time course to assess the impact FoxO1 has on skeletal muscle size.

Our *central hypothesis* is that FoxO1 overexpression impacts skeletal muscle protein synthesis due to suppression of ribosomal biogenesis, thus leading to suppression of skeletal muscle size.

Specific Aims

Aim 1: To assess the impact of FoxO1 overexpression on protein synthesis. Mouse C2C12 skeletal muscle cells have been stably transfected (pBabe retroviral system) with a construct, which results in nuclear translocation of FoxO1 protein upon treatment with tamoxifen (4-OH-T). These cells are grown to confluence and allowed to differentiate into myotubes for four days in low-serum medium. Myotubes are collected after 30 minutes, 60 minutes, and 120 minutes of treatment with tamoxifen (4-OH-T) and insulin (10 μM). Total protein synthesis measurements are determined using radiolabeled isotopes [^{3}H phenylalanine] on control myotubes (EV) and FoxO1 overexpressing myotubes (FoxO1 3A). Hypotheses: i) Protein synthesis is suppressed upon FoxO1 overexpression through the time course when compared to control myotubes. In addition, insulin treatment does not alter protein synthesis in FoxO1 3A myotubes .

Aim 2: To assess the interaction between anabolic signaling and FoxO1 over expression. Retrovirally transfected mouse C2C12 skeletal muscle cells are grown, treated and collected as indicated in Specific Aim 1. Activity of phosphorylated and total levels of Akt and p70^{s6k} will be assessed upon FoxO1 overexpression via western blot. Hypotheses: i) Phosphorylated levels of Akt and p70^{s6k} will be suppressed upon FoxO1 overexpression ii) Total levels of Akt and p70^{s6k} will be unchanged in FoxO1

overexpression iii) Insulin treatment will elicit increases in phosphorylated levels of Akt and $p70^{s6k}$ in control (EV) myotubes, however, FoxO1 3A myotubes will not be altered upon insulin treatment.

Aim 3: To assess the interaction between FoxO1 overexpression and ribosomal biogenesis. Retrovirally transfected mouse C2C12 skeletal muscle cells are grown, treated and collected as indicated in Specific Aim 1. Activity of Cyclin D1 and rpS6 will be assessed via western blot. Hypotheses: i) Cell cycle regulator cyclin D1 protein activity will be suppressed in FoxO1 3A myotubes ii) rpS6 phosphorylation will be suppressed in FoxO1 3A myotubes.

Chapter 2

Literature Review

Skeletal muscle is the most abundant tissue in the human body accounting for 50% of the total body mass.[40,87] It is the major site of metabolic activity and adapts to various physiological demands, including alterations in hormones, amino acids, neural activity, mechanical load, growth factors, nutrient levels, and cytokines.[6,50] In a disuse or catabolic disease state, muscle atrophy occurs and can lead to profound loss of muscle function.[14,17] The loss of muscle function can be debilitating and significantly increases the risk of death in those with diabetes, cancer, and prolonged disuse. According to the CDC[25], 25.8 million people in the United States have diabetes (2011) and the prevalence of diabetes continues to rise each year. Thus, due to the prevalence of diabetes, metabolic syndrome, obesity and frailty associated with muscle dysfunction, it is important to elucidate the molecular mechanisms involved with muscle wasting. The FoxO1 transcription factor is an exciting molecule because it has been implicated not only in the regulation of various cell functions including those related to atrophy, cell cycle progression, apoptosis, but it has also been linked to growth suppression.[48,80]

2.1 FoxO Factors

The Fox proteins are a family of transcription factors that play an important role in regulating the expression of genes involved in various cellular processes including proliferation, differentiation, metabolism, and longevity.[21] The nomenclature for the Fox proteins was originally identified in *Drosophila melanogaster* gene and it was discovered that all Fox proteins contain a 100 amino acid winged helix/forkhead domain.[76] This domain contains three α-helices and two large wing-like loops, hence the name of the winged helix transcription factors.[90, 130] There are 17 different subclasses of Fox proteins (A-Q) ,[61,95] of which the FoxO subclass is studied predominately. The FoxO subclass consists of FoxO1 (forkhead in Rhabdomyosarcomas; FKHR), FoxO3A (FKHR-like protein 1), FoxO4 (acute leukemia fusion gene located in chromosome X; AFX), and FoxO6.[24,56] The FoxO transcription factors are expressed ubiquitously in mammalian tissues, however FoxO1 is studied extensively in skeletal muscle. FoxO1 was first identified in alveolar rhabdomyosarcomas and was identified because of the chromosomal translocation and fusion with Pax 3, resulting in tumor formation.[38] It was identified that 80% of those with alveolar rhabdomyosarcoma contained the FoxO1-Pax3 fusion.

2.1.1 Structure

The structure of FoxO factors include several key features including the DNA binding domain, the nuclear localization signal, the nuclear export sequence, and the

transactivation domain.[95] FoxO factors are controlled via several post-translational modifications including phosphorylation, acetylation, and ubiquitination. In addition, cytoplasmic/nuclear shuttling largely controls the transcriptional activity of FoxO1.[126] The shuttling of proteins is a highly regulated process, in which accessory proteins such as importins and exportins are utilized.[19] Importins and exportins are adaptor proteins, which recognize the specific nuclear localization sequence (NLS) and nuclear export sequence (NES), respectively. Chromosomal region maintenance protein 1 (CRM-1) is an evolutionarily conserved exportin, which recognizes the FoxO1 NES, thus transporting FoxO1 through the nuclear pore complex via Ran-GTP into the cytosol.[28,125] The presence of an NLS or NES is a requirement to remain in the nucleus or cytosol, respectively. Various kinases and proteins modulate the efficiency of the NES and NLS in FoxO1 cytoplasmic/nuclear shuttling.[68]

The main regulator of localization of FoxO1 is phosphorylation. Phosphorylation of FoxO1 prevents nuclear localization and DNA binding to the promoter regions of its gene targets, thereby inhibiting transcription of its gene targets. Akt is the most widely studied kinase that induces cytoplasmic sequestration of FoxO1, thus inhibiting activity.[103,125] Activation of Akt is associated with skeletal muscle growth, in that it is activated by various ligands including insulin, IGF-1, amino acids, and mechanical stimuli.[4] Akt phosphorylates FoxO1 on three highly conserved residues; Thr^{24}, Ser^{256}, and Ser^{319}.[133] Each of these residues differs in functionality and specificity. Akt preferentially phosphorylates Ser^{256}, which is located in the basic region of the C-terminal DNA binding domain. It has been identified that Ser^{256} must first be phosphorylated; followed

by phosphorylation of Thr^{24} and Ser^{319}. It has been demonstrated that Ser^{256} phosphorylation is sufficient to prevent FoxO1 binding to target DNA.[48,138]

In addition to Akt mediated FoxO1 cytoplasmic localization, there are other molecules that have the ability to phosphorylate and inhibit nuclear localization of FoxO1. Serum and glucocorticoid inducible kinase (S6K), Casein kinase 1 (CK1), and Dual specificity tyrosine phosphorylated and regulated kinase 1A (DYRK1A) have the ability to phosphorylate FoxO1 on different sites and sequester FoxO1 in the cytoplasm.[18,104,133] The FoxO1 phosphorylation sites are specific targets of various kinases. Specifically, the phosphorylation of Ser^{256} inhibits transactivation. Phosphorylation of Thr^{24} induces interaction with modular adaptor proteins 14-3-3, which allow for nuclear export of FoxO1.[104] Phosphorylation of Ser^{319} is preferentially targeted by SGK.[18] In addition, the phosphatase PP2A has the ability to dephosphorylate FoxO1, thus causing nuclear localization.[118,135]

2.2 FoxO in skeletal muscle

2.2.1 Skeletal muscle proliferation

The FoxO1 transcription factor is an exciting molecule because it has been implicated not only in the regulation of various cell functions including cell cycle progression and apoptosis, but has also been linked to growth suppression.[40,46,50,62,80,96] The activation of FoxO1 in mammalian cells often causes strong inhibition of cellular proliferation. The antiproliferative effect of FoxO1 can oppose the growth stimulating

effects of various mitogens associated with Akt pathway activation. Often, the antiproliferative effect is due to increased activity of cell cycle inhibitors and increased apoptosis.[21] Specifically, FoxO1 induces the transcription of cyclin dependent kinase inhibitor (CDK inhibitors) $p27^{kip1}$ and $p21^{cip1}$.[115] It has been demonstrated that increased protein expression of p27 or p21 allows for inhibition of Cyclin E/CDK2 and Cyclin D1/CDK4 complexes respectively, thus inhibiting progression of the cell cycle through the S-phase or G1 phase. FoxO1 has the ability to transcriptionally repress Cyclin D, which induces cell cycle arrest at the G1 phase.[41] Mammalian cyclins are classified into 12 different types, named from A to I, based on structure and function. Cyclin D1 is the key cyclin regulator of the G1 phase and it binds to and activates CDK 4/6 in the cell cycle. In cells that have the ability to proliferate, FoxO1 activation functions to promote cell cycle arrest in the G1/S phase, thus supporting its role as a tumor suppressor gene.[15,115]

2.2.2 Skeletal muscle differentiation

The differentiation and maturation of skeletal muscles requires a unique milieu between signaling pathways and regulatory networks of myogenic transcription factors. Unlike proliferation, the environment of differentiation differs in that differentiation involves withdrawal from the cell cycle. Also, unlike most peptide growth factors that induce proliferation, insulin-like growth factors (IGF-1) induce differentiation in vivo and in vitro via a P13k-Akt dependent fashion.[131] In addition, several cell cycle regulators are necessary for differentiation in skeletal muscle.[67] One of the key steps in

skeletal muscle differentiation involves down regulation of cell cycle machinery, such as cyclin-dependent kinases (CDK's) and cyclins. In addition, activation of various cell cycle inhibitors such as retinoblastoma (Rb), $p21^{cip1}$, $p27^{kip1}$, and Rb associated proteins (p130) are also associated with differentiation of myoblasts.[67,117] The tumor suppressor protein retinoblastoma (Rb) is necessary for skeletal muscle differentiation. Specifically, Rb induces and maintains permanent cell cycle withdrawal through regulation of E2F genes.[30,89] According to Zackenhaus et al,[136] insufficient phosphorylation of Rb results in failure of myogenesis due to apoptosis of myoblasts, as well as defective formation of myotubes. Thus, coordination of cell cycle machinery and Rb is essential for proper myogenesis.

The role of FoxO1 in skeletal muscle differentiation is complex. In primary mouse myoblasts, it has been demonstrated that within 48 hours of serum removal, FoxO1 translocates to the nucleus.[16] However, if FoxO1 resides in the nucleus during late stage differentiation, increased proteasome activity and apoptosis occurs.[53] In fact, FoxO1 translocation into the nucleus enhances the rate of myotube fusion at the onset of differentiation, however FoxO1 nuclear localization during the later stages of differentiation has the opposite effect on fusion.[16]

2.2.3 Skeletal Muscle Atrophy

Skeletal muscle is a highly adaptable organ and its functionality is dependent upon the fine balance between protein degradation and protein synthesis.[111] Degradation of skeletal muscle has a critical impact upon functionality, rehabilitation, disease

prognosis as well as metabolism, as skeletal muscle acts as the major glucose uptake organ in the body. In an atrophic condition, proteolytic systems are activated, and contractile apparatus are degraded, thus leading to loss of muscle mass.[12] In fact, breakdown of myofibrillar proteins is the primary cause of loss of fiber mass.[110] FoxO1 activation is sufficient to activate the ubiquitin proteasome system (UPS), as well as a variety of proapoptotic genes.[80,96] In mouse models in which FoxO1 has been overexpressed specifically in skeletal muscle, smaller muscle mass is noted and blunting of hypertrophy is demonstrated upon mechanical loading.[62,96] In addition to characteristically small muscles, increased expression of E3 ubiquitin ligases MAFbx/atrogin-1 and MuRF-1 are demonstrated.[102] In addition, in models of cachexia, denervation, disuse, and nutritional deficit, the activation of FoxO1 has been identified. The fundamental role of FoxO1 in all types of skeletal muscle atrophy has been established, however, it is unknown whether FoxO1 has a role in suppression of protein synthesis.

2.2.4 Akt Signaling

The canonical Akt/mTOR pathway has been shown to be an important regulator of postnatal skeletal muscle size.[14,40] At the top of the signaling cascade is the Insulin Receptor that is activated upon binding of its ligand, which is insulin, or IGF-1. Upon ligand binding, the tyrosine kinase Insulin receptor is autophosphorylated and activated. This allows for IRS-1 tyrosine phosphorylation, which phosphorylates PIP-2 to PIP-3 in the plasma membrane. Akt will translocate to the plasma membrane and PDK-1 will

phosphorylate and activate Akt on the Serine^{473} and Threonine^{308} sites.[128] Akt is involved in many cellular processes including cellular proliferation, cellular growth, protein synthesis, and glucose uptake.[14] Akt sequentially phosphorylates and inhibits TSC1/TSC2 complex (Hamartin/Tuberin complex).[26,93] Inhibition of TSC1/TSC2 allows for the small GTPase molecule called Rheb to be active in its GTP bound state. Rheb will physically interact with and activate mTORC.[60,71,33,57] mTOR is known as mammalian target of rapamycin and was originally identified as the physical target of the drug rapamycin, in which rapamycin inhibits mTORC activity. mTORC is strongly regulated by nutrient availability and cellular energy levels and is a major consumer of energy.[60,71,60] Recently, Chen et al[26] demonstrated that a novel function of FoxO1 includes that of energy homeostasis. In times of physiological stress, FoxO1 maintains high Akt activity and suppresses mTORC activity, in order to maintain cellular energy balance. Downstream of mTOR is p70^{s6k}, which has been demonstrated to be associated with regulation of protein synthesis.[7,4,5] p70^{s6k} phosphorylates ribosomal proteins S6 and S17, which induces 5' TOP mRNA translation.[86] 4EBP-1 is another molecule that has been demonstrated to regulate protein synthesis, in that phosphorylation of 4EBP-1 leads to the release of eIF4E. The eIF4 family is one of the critical regulatory elements governing mRNA translation initiation. The release of 4EBP-1 from eIF4E allows for interaction with eIF4G and eIF4A, which form the eIF4F complex and induces translation initiation. However, it has been demonstrated that blocking the formation of eIF4F has no effect on mRNA translation initiation or protein synthesis.[55] Therefore, the role of 4EBP-1 in mRNA translation initiation is controversial. Another molecule that plays a strong role in the regulation of protein synthesis is phosphorylated heat-acid

stabled protein (PHAS-1). PHAS-1 phosphorylation frees eIF4E and stimulates translation initiation. PHAS-1 has been shown to respond to insulin treatment resulting in increased protein synthesis and PHAS-1 has been shown to be regulated by $p70^{s6k}$.[72,77] Therefore, the regulation of muscle growth through the context of the canonical Akt/mTOR pathway is complex and controversial. It is necessary to digress from the evaluation of this pathway in order to better understand the complex regulation of skeletal muscle growth.

2.2.5 Ribosome Biogenesis

During periods of growth, feeding stimulates protein synthesis in all tissues, and the highest response occurs in skeletal muscle.[20,29] IGF-1, insulin, and amino acids are the key players in stimulation of protein synthesis.[65,122,127] Protein synthesis is a complex process in which there are three distinct stages. The stages of protein synthesis include: Initiation, Elongation, and Termination. The stages of protein synthesis involve the ribosome, tRNAs, additional proteins, and aminoacyl tRNA synthetases.[64] Initiation begins with binding of initiator methionyl-tRNA (met-tRNA) to the 40S ribosomal subunit,[98] which is mediated by the eukaryotic initiation factor (eIF2). Following binding of met-tRNAi to the ribosome, the mRNA strand will bind to the met-tRNA complex, which is mediated by the eIF4 complex of proteins.[120] Ribosomes are complex units composed of two subunits: large (60S) and small (40S). The 40S subunit is made up of a single RNA molecule, 18S ribosomal RNA and 33 proteins, whereas the large 60S subunit contains 3 distinct rRNA molecules referred to as 5S, 5.8S, and 28S rRNA and 46

associated proteins.[82] The rRNA is synthesized as precursor rRNA molecules and undergoes extensive post-transcriptional modifications to produce mature and functional rRNA.[107] rRNA are synthesized by RNA Pol I and III, while RNA Pol II synthesizes mRNA coding for ribosomal proteins.[92] Although there are many ribosomal proteins, one of the most widely studied ribosomal proteins is rpS6. rpS6 is one of the only ribosomal proteins identified to undergo phosphorylation and it has been shown to respond to insulin [119], leucine [66], glucose [42], IGF-1 [49], and various other amino acids.[123] The phosphorylation sites of rpS6 include that of Ser^{235}, Ser^{236}, Ser^{240}, Ser^{244}, and Ser^{247} with phosphorylation beginning at Ser^{236} and continuing with Ser^{235}, Ser^{240}, Ser^{244}, and Ser^{247} sequentially for complete activation.[82] Ruvinsky et al[108] utilized a knock-in mouse to mutate all phosphorylation sites to alanine, in order to better understand the role of rpS6 phosphorylation in regulating protein synthesis. Mice with the genetic knock-in displayed reduced overall size and skeletal muscle weakness, which is a trait shown by $p70^{s6k}$ deficient mice, as well. This indicates that phosphorylation of rpS6 is associated with increased cell growth.[49,66,82,109] Surprisingly, protein synthesis rates were not globally suppressed in the knock-in mice or in mouse embryonic fibroblasts (MEF) upon loss of phosphorylation of rpS6.[108,109] However, various studies have demonstrated that polysomes contain a greater amount of phosphorylated rpS6 than subpolysomes, indicating the critical role of phosphorylation of rpS6 in regulating protein translation initiation.[34,124]

Skeletal muscle hypertrophy is characterized by increases in muscle mass, protein synthesis rates and total RNA content. Increases in total RNA is indicative of increases in ribosomal RNA (rRNA).[87] Recently, in a chronic overload model in mice, it has been

demonstrated that skeletal muscle hypertrophy is the result of both increased protein synthesis and increased proteasome activity. The increased proteasome activity was associated with increases in the MAFbx/atrogin-1, MuRF-1, as well as FoxO1 and FoxO3a activity. In addition, it was demonstrated that mere activation of FoxO1 is sufficient to increase proteasome activity upon mechanical overload.[9]

2.3 FoxO and ER Stress

The endoplasmic reticulum (ER) is where folding and posttranslational modifications to proteins occur. Certain conditions, including glucose deprivation or hyperlipidemia can lead to accumulation of unfolded proteins in the ER lumen[137], thus preceding the unfolded protein response (UPR).[70,78,106,134] In the UPR there are three main proteins that are involved: ATF6 (activating transcription factor 6), IRE1alpha (inositol-requiring enzyme 1alpha), and PERK (protein kinase R-like ER protein kinase). In a basal state, these proteins associate with chaperone protein called BiP/GRP78 (binding protein/glucose-regulated protein-78).[74] BiP/GRP78 is known as a central regulator for ER stress and acts through a bind-release mechanism to regulate the transmembrane ER stress regulators (ATF6, PERK, IRE1alpha). Upon ER stress or accumulation of unfolded proteins, the ER stress regulators are released and activate genes such as CHOP (CCAAT/enhancer binding protein homologous protein) and ATF4.[31] These genes result in the activation of apoptotic machinery in that Bcl-2 expression is downregulated and Bim is upregulated.[100] Bim is responsible for

suppression of antiapoptotic factors such as Bcl-2. Therefore, the UPR and ER stress results in the induction of apoptosis, as well as the suppression of protein synthesis.

2.4 Concluding Remarks

The purpose of the present study was to develop a model in which hypertrophic stimuli in the form of insulin is utilized to study the interaction of FoxO1 with protein synthesis, cell cycle machinery and ribosome biogenesis. This is conceptually based on previous studies in which both myotube hypertrophy[127] and increases in total RNA[139] have been shown to be induced by in vitro insulin treatment. In addition, it was hypothesized that insulin stimulation will result in myotube hypertrophy characterized by 1) increased total protein content per well, and 2) increased protein synthesis. Further, it was hypothesized that these effects will be blunted in FoxO1 3A myotubes. Although the role of FoxO1 in upregulation of proteolysis is well characterized, it is important to understand the role of FoxO1 in protein synthesis and ribosome biogenesis mechanisms, as well. As recently demonstrated, skeletal muscle hypertrophy is the result of increased protein synthesis and increased protein degradation.[9] Thus, it is imperative to delineate the role of FoxO1 in regulation of ribosome biogenesis and protein synthesis. The knowledge serves as the basis for new discoveries and to establish the significance of FoxO1 in muscle wasting disorders.

Chapter 3

Experimental Methods

3.1 Retroviral Transfection

FoxO1-estrogen receptor (FoxO1-ER 3A) fusion proteins that are treated and activated with 4-hydroxytamoxifen (4-OH-T) were stably expressed in C2C12 skeletal myoblasts using a pBABE retroviral system, as previously reported.[80] The FoxO1-ER 3A fusion protein construct was co-transfected together with vectors expressing gag-pol, REV, and VSV-G into 293FT cells (Invitrogen, Carlsbad, CA) to generate a third generation lentiviral construct. Transfection was achieved using Lipofectamine 2000 (Invitrogen) using 100 ng total DNA per cm^2 of the growth plate. The supernatants were harvested and the cell debris was removed by centrifugation at 2000 x g. C2C12 cells were seeded in flat bottom culture plates and grown to 50% confluent. The supernatant of the virus and polybrene (5 ng/ml, Sigma-Aldrich, St. Louis, MO) was used to infect C2C12 cells to establish a cell line that has been stably regulated. After 72 hours, the cells were positively selected by puromycin (Specialty Media, Temecula, CA; 3.5 μg/ml) for 24 hours. The FoxO1 3A construct corresponds to three alanine mutations of FoxO1 that Akt preferentially phosphorylates; Ser ^{319}, Ser ^{256}, and Thr ^{24}. The control cells have

been stably transfected with a pBABE empty vector (EV) construct [empty cassette (no FoxO1-ER expression)].

3.2 Cell Culture

The FoxO1 3A cells, the control (EV) cells, and the C2C12 cells were utilized at passage 6 for all cell culture experiments. Myoblasts were seeded on 6 well plates in a warm humidified environment (37°C; 5% CO2) and bathed in skeletal muscle growth medium (Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum and gentamicin). Growth medium was changed 1 d after seeding and 48 h thereafter until cells reached 90% confluence. When myoblasts reached 90% confluence, growth medium was replaced with differentiation medium (DMEM; Sigma-Aldrich) supplemented with 2% heat inactivated horse serum (Invitrogen) and gentamicin (Sigma) to induce differentiation into myotubes. Differentiation medium was changed every 48 h for 4 d.

3.3 Treatment of Myotubes

On day 4 after initiation of differentiation, myotubes were washed (2X) with warmed PBS and bathed in DMEM for 2 h. Myotubes were then treated with insulin treatment (10 μM) or no treatment for a predetermined time course consisting of 30 minutes, 60 minutes, and 120 minutes of treatment. Myotubes were also collected at a pre treatment time point, which consisted of no treatment. The myotubes that incurred no

insulin treatment received treatment with an HCL (10 μM) vehicle treatment, for control purposes. Upon insulin treatment, all of the myotubes were also subjected to treatment with 4-hydroxytamoxifen (4-OH-T), which drives the mutated FoxO1 protein into the nucleus. Myotubes were homogenized in NP-40 buffer or RIPA buffer supplemented with HALT protease inhibitors single-use cocktail (Thermo Scientific, Rockford, IL) and 10 mM Na_3VO_4 and scraped from the cell culture plate. Homogenates were lysed using a cell sonicator and centrifuged at 14,000 x g for 10 minutes at 4°C and rapidly frozen at -80°C. The protein concentrations were analyzed by using the Bio-Rad DC protein assay kit (Hercules, CA) and quantified using spectrophotometry on a microtiter plate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA).

3.4 Protein Synthesis

Protein synthesis rates (fmoles/mg of protein/hr) were measured after time course treatment of the myotubes. After treatment with 4-OH-T and insulin or vehicle, myotubes were incubated for 1 hour in DMEM containing 2.5 mM phenylalanine and 10 μCi/ml [^{3}H] phenylalanine (Perkin Elmer) to ensure equilibration of intracellular and extracellular specific radioactivities.[39,47,52] After incubation, the myotubes were washed 3 times with ice-cold PBS (pH 7.5) and homogenized by 10% trichloroacetic acid (TCA). A portion of the culture medium was saved to determine specific activity of the medium (c.p.m) during pilot experiments. TCA homogenates were incubated on ice for 30 minutes, plates were thoroughly scraped, followed by centrifugation at 4500 x g for 5 min. The supernatant was discarded and the TCA insoluble pellet was resuspended in

10% TCA, followed by centrifugation at 4500 x g for 5 min. This was repeated three consecutive times, and the TCA insoluble material was dissolved in 0.15 M NaOH at 55° C for 1 hour with frequent vortex mixing. Aliquots of each sample were analyzed to determine counts per minute via liquid scintillation counting and DC protein assay (Bio-Rad). The rate of protein synthesis was calculated as *fmoles of [^{3}H] phenylalanine/mg of protein/hr*.

3.5 Western Blotting

Protein homogenates (30 μg) were solubilized in Laemmali buffer and heated at 37° C for 15 minutes. Subsequently, the samples were resolved by SDS-PAGE on 10% tricine gels, transferred to nitrocellulose membrane via semi dry blotting transfer (TransBlot Transfer Cell, Bio-Rad Laboratories, Inc., Hercules, CA) for 30 minutes at 20 V constant. Following the transfer, the membranes were blocked in 5% non-fat dry milk in TBS for 1 hour at room temperature, and immunoblotted overnight at 4° C with phospho-Akt (Ser^{473}), total Akt, phospho-$p70^{s6k}$ (Thr^{389}), total $p70^{s6k}$, phospho-Ribosomal Protein S6 ($Ser^{235/236}$), and Cyclin D1 (1:1000 for phospho and total Akt and p70s6k; 1:20,000 for phospho-rpS6; 1:2,000 for Cyclin D1; Cell Signaling Technology, Beverly, MA). Equal protein loading was verified using the GAPDH antibody (1:20,000; Cell Signaling Technology). After 1-hour incubation with an infrared-conjugated Alexa Flour 680 secondary antibody (1:5000; Molecular Probes, Carlsbad, CA) at room temperature, the proteins were analyzed via infrared detection and quantified using densitometry.

3.6 Statistical Analysis

Statistical Analyses were performed using SPSS. A one-way factorial analysis of variance (ANOVA) was utilized to determine differences in total FoxO1 protein expression and total protein content. All other analyses used a two-way ANOVA ($P \leq 0.05$) to determine differences between conditions (Cell type [control vs. FoxO1 3A] x time [pre, 30 min, 60 min, 120 min.]) for all dependent variables. Student-Newman-Keuls post hoc analyses ($P \leq 0.05$) were used to locate differences between means if a significant main effect was found. Data are reported as mean ± SEM.

Chapter 4

Results

To establish the impact that FoxO1 has on skeletal muscle protein synthesis and ribosomal activation, a stably transfected cell culture model was developed in which FoxO1 estrogen receptor fusion proteins were infected into C2C12 cells, as previously described. As shown in Figure 4-1, total FoxO1 protein in FoxO1 3A cells compared to control cells was significantly elevated prior to treatment with 4-hydroxytamoxifen (4-OH-T). Additionally, nuclear localization of the FoxO1 fusion protein, upon 4-OH-T treatment, has been demonstrated in our model previously.[10,85] Thus, our model sufficiently overexpressed total FoxO1 protein, as shown in Figure 4-1, and nuclear localization of FoxO1 protein occurred upon 4-OH-T treatment. The tight regulation of FoxO1 by 4-OH-T renders it possible to study the acute modulation of FoxO1 function and its impact on skeletal muscle protein synthesis.

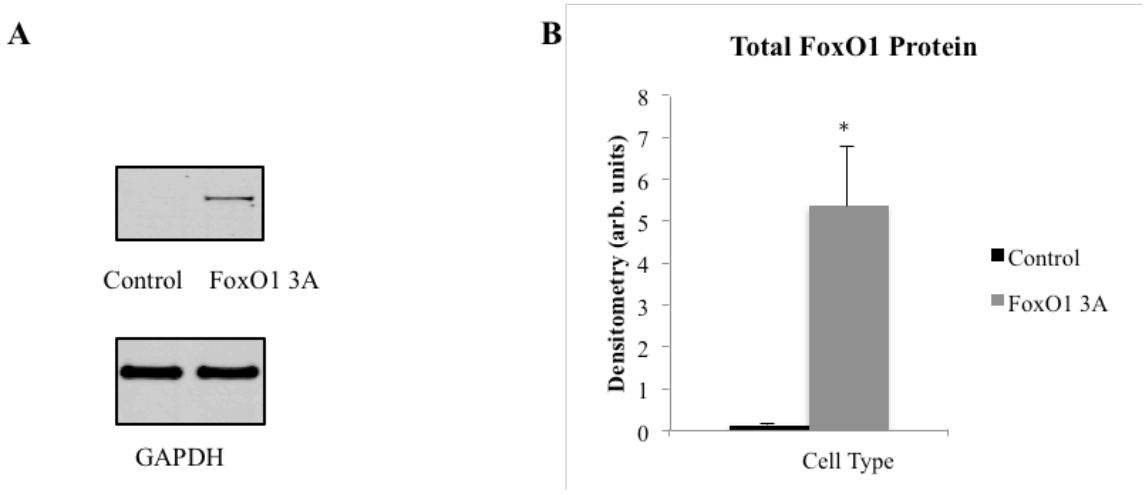

Figure 4-1: Total FoxO1 protein expression. Panel A: Representative western blot of total FoxO1 protein expression in control and FoxO1 3A myotubes. GAPDH is representative of total protein loading. Panel B: Quantification of Total FoxO1 protein expression. *, FoxO1 3A is significantly higher than control. n=3 for all groups.

FoxO1 overexpression inhibits skeletal muscle protein synthesis.

In order to evaluate skeletal muscle protein synthesis, the incorporation of [^{3}H] phenylalanine per milligram of protein (fmoles/mg/hr) and total protein content (mg/well) was quantified in FoxO1 3A and control myotubes over an acute time course. As shown in Figure

4-2, no significant differences in total protein content were observed in control and FoxO1 3A myotubes across the time course. Additionally, treatment with insulin did not elicit differences in total protein content between cell types. The lack of differences in total protein between cell types indicates that any alterations in protein synthesis occurred prior to any phenotypic alterations in protein content in the myotubes. As supported throughout the literature, FoxO1 plays a prominent role in initiating muscle atrophy machinery prior to any phenotypic alterations.[110] In Figure 4-3, protein synthesis

significantly decreased in FoxO1 3A myotubes after 120 minutes without insulin treatment ($p<.001$) and with insulin treatment ($p<.001$), when compared to control. Nuclear translocation of FoxO1, upon administration of 4-OH-T, resulted in suppression of protein synthesis by 64% and this suppression of protein synthesis was not rescued after 120 minutes of insulin treatment. Therefore, these findings demonstrated that suppression of protein synthesis occurred in a robust manner upon FoxO1 overexpression and insulin treatment was not able to rescue this robust suppression. Additionally, these data support the concept that FoxO1 initiates muscle atrophy machinery through suppression of protein synthesis, prior to any phenotypic loss of protein content.

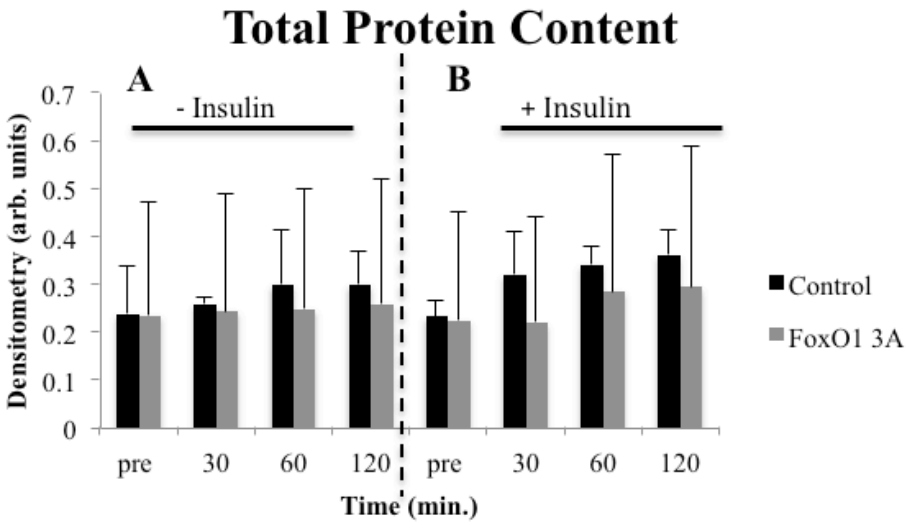

Figure 4-2: Total protein content for control and FoxO1 3A myotubes. Panel A: Quantification of total protein content (mg/well) in control and FoxO1 3A myotubes without insulin (- Insulin). Panel B: Quantification of total protein content (mg/well) in control and FoxO1 3A myotubes with insulin treatment (+ Insulin). n=6 for all groups. Data represented as means ± SEM.

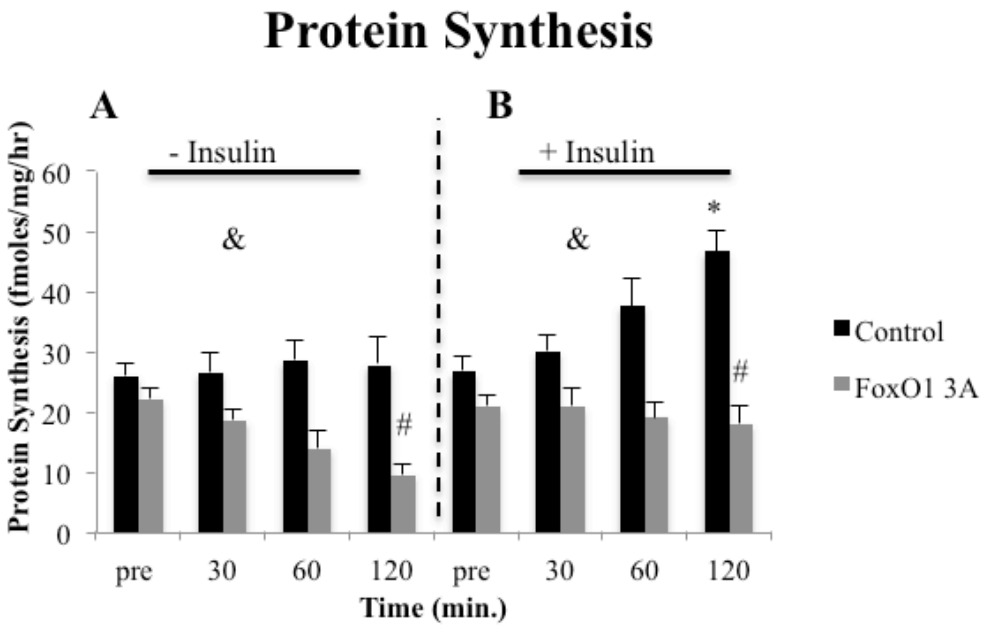

Figure 4-3: Protein synthesis for control and FoxO1 3A myotubes. Quantification of total protein synthesis via (^{3}H) phenylalanine uptake (fmoles/mg/hr). Panel A: Representative of control and FoxO1 3A myotubes without insulin treatment (- Insulin). Panel B: Representative of control and FoxO1 3A myotubes with insulin treatment (+ Insulin). &, significantly higher in control myotubes than FoxO1 3A regardless of time; *, significant difference from pre time point; #, significant difference in FoxO1 3A myotubes at 120 minutes compared to control myotubes. $P<0.05$ for all significant differences. n=6 for all groups. Data represented as means ± SEM.

FoxO1 mediated inhibition of skeletal muscle growth was associated with alterations of Akt signaling.

Akt is a purported anabolic molecule that plays a prominent role in the regulation of skeletal muscle hypertrophy, particularly after insulin treatment.[14] Phosphorylation of Akt often signals increased activity, thus the phosphorylation status of this anabolic

marker was analyzed. As shown in Figure 4-4, Akt phosphorylation (Ser^{473}) was significantly elevated in control myotubes compared to FoxO1 3A counterparts ($p<.001$). In addition, insulin treatment resulted in a significant elevation of Akt phosphorylation in control myotubes compared to FoxO1 3A myotubes ($p=.002$). Increased phosphorylation of Akt after insulin treatment in control conditions is corroborated throughout the literature, therefore this finding was not surprising.[1,84,88,97,98] However, the surprising finding to note was that FoxO1 3A myotubes do not demonstrate increased Akt phosphorylation to the same degree as control, specifically upon insulin treatment. Therefore, Akt activity is blunted in the FoxO1 3A myotubes, both during basal conditions and with insulin treatment. In regards to total Akt protein expression, the control myotubes demonstrated increased total Akt protein both without and with insulin treatment (Figure 4-3). However, when taken together, the alterations in phosphorylated Akt indicate blunted activity in the FoxO1 3A model. These data, taken alongside the data in the literature, corroborates the suppression of Akt activity in the FoxO1 3A model.

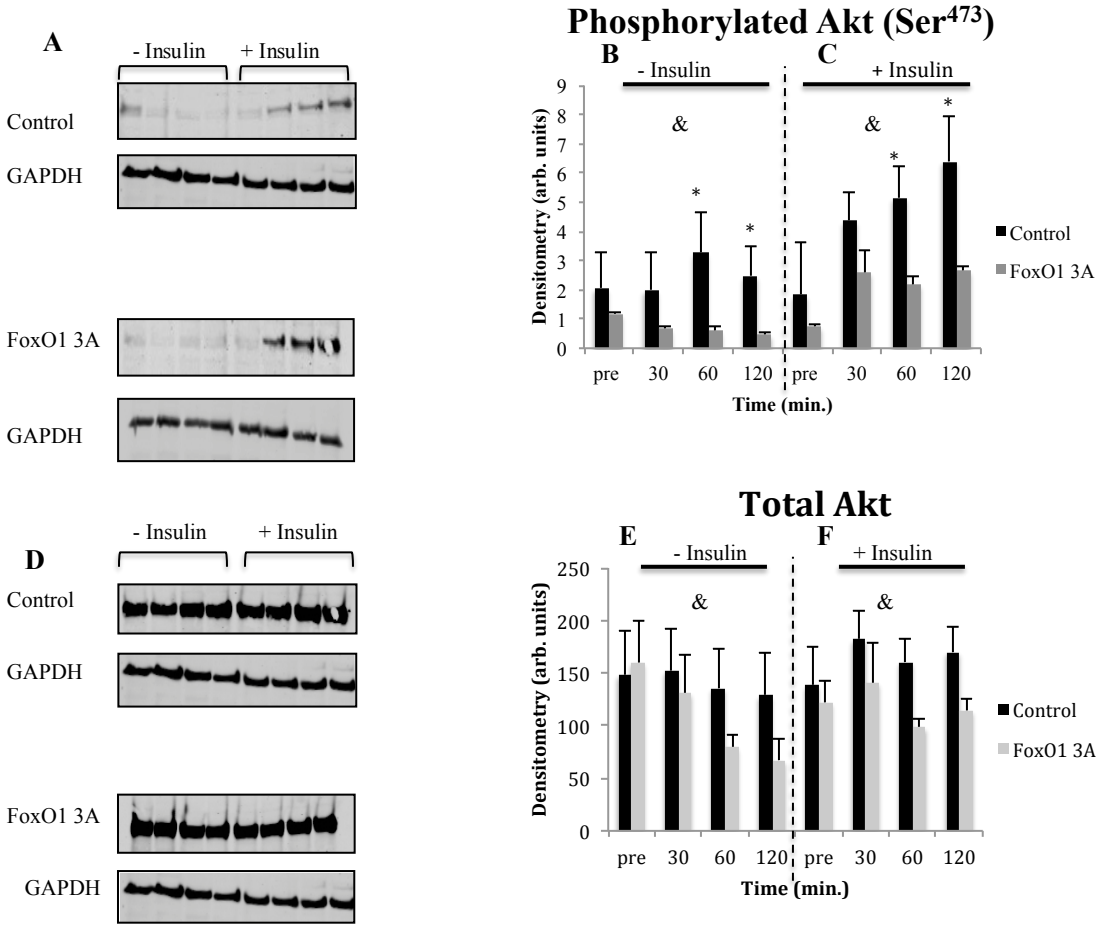

Figure 4-4: Phosphorylated and Total Akt in control and FoxO1 3A myotubes. Panel A and D: Representative western blot analyses of control and FoxO1 3A myotubes through the time course without and with insulin treatment (10μM), as indicated. GAPDH is representative of total protein loading. Panels B and E: Quantification of western blot data for phosphorylated and total Akt without insulin (- Insulin), respectively. Panels C and F: Quantification of western blot data for phosphorylated and total Akt with insulin (+ Insulin). &, significantly higher in control myotubes than FoxO1 3A regardless of time; *, significant difference from pre time point. $P<0.05$ for all significant differences. n=6 for all groups. Data represented as means ± SEM.

FoxO1 mediated inhibition of skeletal muscle protein synthesis occurred independently of alterations in $p70^{s6k}$ signaling.

Throughout the literature, $p70^{s6k}$ has been identified to have increased activity upon treatment with an anabolic stimulus, such as resistance exercise or leucine administration.[51,63] Moreover, $p70^{s6k}$ has been linked to increased cell growth and myotube hypertrophy and has been coined to do so by increasing mRNA translation initiation. Thus, the phosphorylation status of $p70^{s6k}$ was analyzed in order to better understand the role FoxO1 plays in regulating ribosomal activation. As shown in Figure 4-5, basal levels of phosphorylated $p70^{s6k}$ were elevated in control myotubes compared to FoxO1 3A myotubes ($p=.014$). However, insulin treatment resulted in no significant differences in phosphorylation of $p70^{s6k}$. In contrast, basal levels of total $p70^{s6k}$ were suppressed in control myotubes compared to FoxO1 3A counterparts ($p=.022$). However, no differences were noted in levels of total $p70^{s6k}$ upon insulin treatment. Further, there were no interaction effects between cell types and time course noted for either phosphorylated or total levels of $p70^{s6k}$. Although there were alterations in total and phosphorylated levels of $p70^{s6k}$ between control and FoxO1 3A myotubes, treatment with insulin did not modify these alterations. Therefore, this data demonstrated that insulin treatment did not elicit increased $p70^{s6k}$ activity in either control or FoxO1 3A myotubes. Opposite our hypothesis, the alterations in protein synthesis did not appear to be due to suppression of $p70^{s6k}$ activity, as indicated in the data.

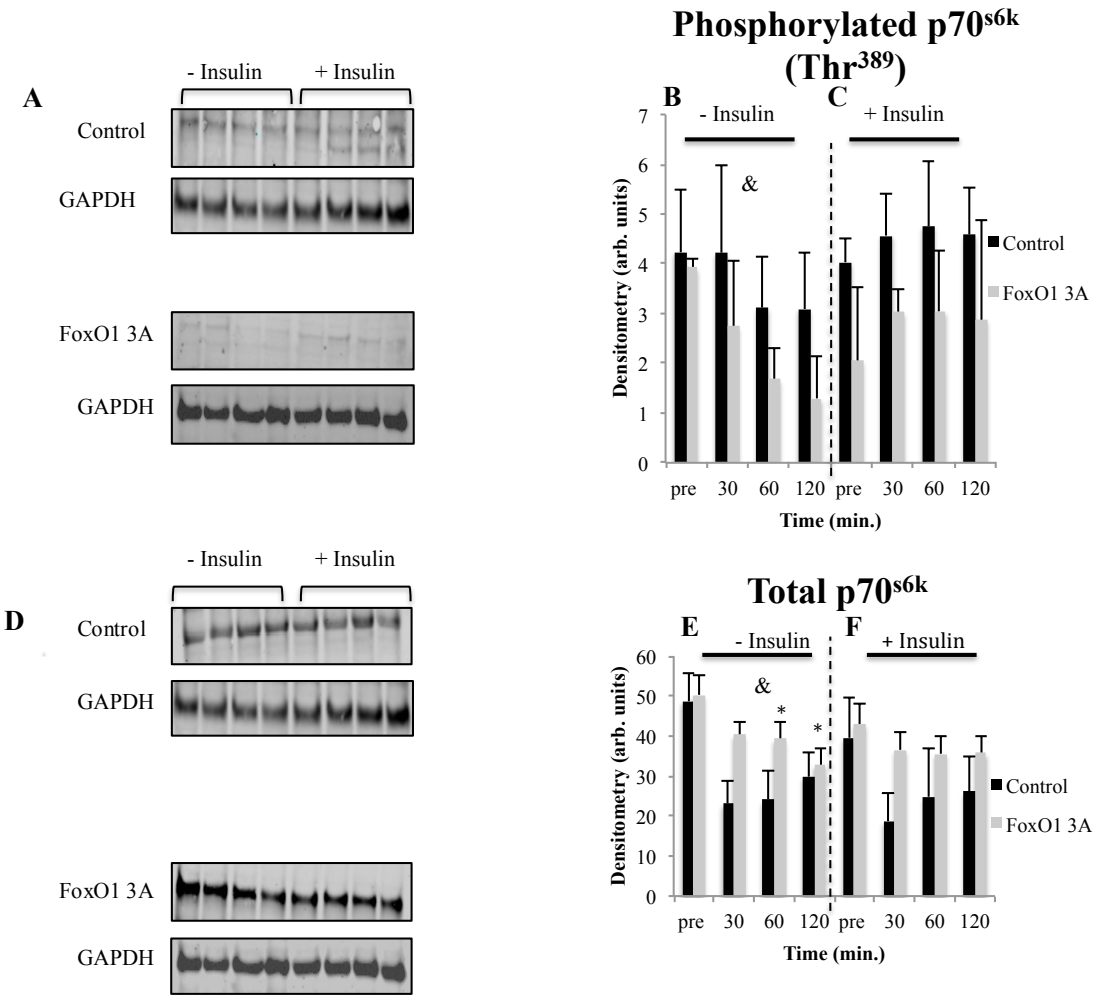

Figure 4-5: Phosphorylated and total p70^{s6k} protein in control and FoxO1 3A myotubes. Panels A and D: Representative western blot analyses from control and FoxO1 3A myotubes through the time course without and with insulin treatment (10μM), as indicated. GAPDH is representative of total protein loading. Panels B and E: Quantification of western blot data for phosphorylated and total p70^{s6k} without insulin treatment (- Insulin), respectively. Panels C and F: Quantification of western blot data for phosphorylated and total p70^{s6k} with insulin treatment (+ Insulin), respectively. &, significantly higher value for cell type, regardless of time; *, significant difference from pre time point. $P<0.05$ for all significant differences. n=6 for all groups. Data represented as means ± SEM.

FoxO1 mediated inhibition of skeletal muscle growth was associated with alterations in ribosomal protein S6 expression.

Throughout the literature, the ribosomal protein S6 (rpS6) has been widely associated with increased mRNA translation initiation.[34] Therefore, in order to evaluate ribosomal activation, the phosphorylation status of rpS6 was quantified. As shown in Figure 4-6, there were alterations in phosphorylation of rpS6. Phosphorylation of rpS6 was significantly elevated at 120 minutes basally in control myotubes compared to FoxO1 3A counterparts ($p=.049$). Additionally, insulin treatment resulted in further elevations in phosphorylation of rpS6 in control myotubes and these elevations were not mirrored in the FoxO1 3A myotubes ($p<.001$). Interestingly, this data demonstrated that FoxO1 3A myotubes suppressed the phosphorylation of rpS6 and this suppression was not rescued after 120 minutes of insulin treatment**.**

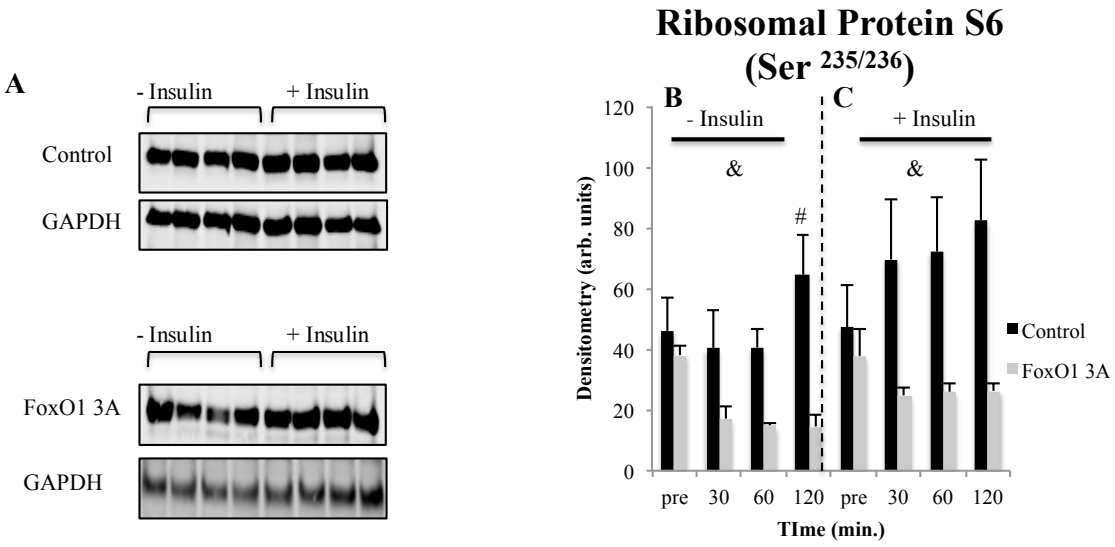

Figure 4-6: Phosphorylated ribosomal protein S6 in control and FoxO1 3A myotubes. Panel A: Representative western blot analyses from control and FoxO1 3A myotubes through the time course without and with insulin treatment, as indicated. GAPDH is representative of total protein loading. Panel B: Quantification of western blot data for phosphorylated rpS6 without insulin (- Insulin). Panel C: Quantification of western blot data for phosphorylated rpS6 with insulin (+ Insulin). &, significantly higher in control myotubes than FoxO1 3A regardless of time; #, significantly higher in control myotubes at 120 minutes compared to FoxO1 3A. $P<0.05$ for all significant differences. n=6 for all groups. Data represented as means ± SEM.

FoxO1 mediated inhibition of protein synthesis was not associated with alterations in Cyclin D1 protein expression.

The potent cell cycle regulator, Cyclin D1, has been identified in the literature to increase upon serum stimulation and has been tied to myotube hypertrophy, in vitro.[87] Therefore, in order to identify any potential deficiencies in cell cycle regulation, Cyclin D1 protein expression was analyzed. As shown in Figure 4-7, Cyclin D1 protein

expression was not altered between the cell types or with insulin treatment. This data demonstrated that FoxO1 3A myotubes did not alter protein expression of Cyclin D1. Therefore, it can be concluded that protein synthesis suppression in FoxO1 3A myotubes was not due to alterations in cell cycle machinery associated with Cyclin D1 protein expression.

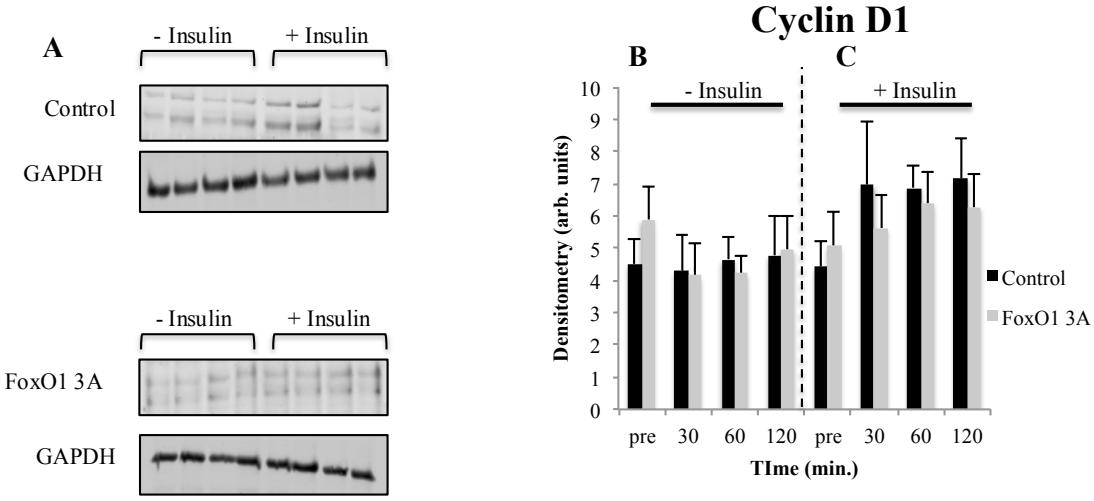

Figure 4-7: Cyclin D1 protein expression in control and FoxO1 3A myotubes. Panel A: Representative western blot analyses from control and FoxO1 3A myotubes through the time course without and with insulin treatment. GAPDH is representative of total protein loading. Panel B: Quantification of western blot data for Cyclin D1 without insulin (- Insulin). Panel C: Quantification of western blot data for Cyclin D1 with insulin (+ Insulin). n=6 for all groups. Data represented as means ± SEM.

Chapter 5

Discussion

The FoxO family is integral to various processes including regulation of cell survival, cell cycle, and cellular proliferation.[99] In addition, FoxO1 plays a strong role in regulating skeletal muscle growth. Transcriptional targets of FoxO1 include those associated with muscle atrophy. Ample research has focused on the impact of FoxO1 on muscle atrophy and it has been established that FoxO1 increases activity of E3 ubiquitin ligases, thus leading to marked protein degradation and loss of muscle mass. Additionally, it has been demonstrated that FoxO1 is highly involved in all types of skeletal muscle atrophy including denervation, disuse, cachexia, sepsis, and nutritional deficit.[110] Also, there is considerable evidence that all types of skeletal muscle atrophy are associated with increased protein degradation and apoptosis. Moreover, atrophying muscles show increased mRNA and protein expression of the classic "atrogenes" known as MAFbx/atrogin-1 and MuRF-1. Specifically, increased ubiquitin conjugated proteins, enhanced rates of ubiquitination, and increased FoxO1 nuclear activity has been widely demonstrated in various models of skeletal muscle atrophy.[81,110,132] For example, denervation associated skeletal muscle atrophy is quelled in rodent models in which

MAFbx/atrogin-1 is knocked out, thus indicating the importance of the "atrogenes".[13] In addition, it has been shown that FoxO signaling is necessary and required for physiological muscle atrophy.[116] In fact, inhibition of transcriptional activity of FoxO prevents muscle atrophy and induces muscle hypertrophy in vivo.[102] Thus, the literature indicates the strong role that FoxO1 plays in regulating skeletal muscle atrophy. However, little is known about the impact FoxO1 plays on regulating loss of muscle mass through alterations in protein synthesis.

To this end, we have developed an inducible model of skeletal muscle atrophy to elucidate the impact of FoxO1 on the regulation of anabolic markers, protein synthesis and proteins associated with ribosomal activation. The overall objectives of this study were to characterize the impact between FoxO1 overexpression and protein synthesis in vitro. The findings from this study demonstrated that skeletal muscle protein synthesis was suppressed upon FoxO1 overexpression and this suppression was not rescued after 120 minutes of insulin treatment. In fact, the suppression of protein synthesis occurred prior to any discernible loss of total protein content. Although suppression of Akt activity was associated with alterations of protein synthesis, we found it important to note that there is no robust change in activity of $p70^{s6k}$. Rather, the major findings of this study indicated that suppression of protein synthesis, upon FoxO1 overexpression, occurred concomitantly with suppression of phosphorylation of rpS6. This demonstrated that FoxO1 mediated suppression of protein synthesis occurred despite intact signaling through $p70^{s6k}$ and may be directly related to mechanisms involving rpS6 and ribosomal activation.

Strong evidence linking FoxO1 expression to skeletal muscle atrophy through the downregulation of Akt and $p70^{s6k}$ has been provided through the use of in vivo and in vitro models.[14,23,101,105,112,114,121] Our findings demonstrated that the phosphorylation of Akt was, in fact, suppressed upon FoxO1 overexpression. Additionally, there was a significant elevation in phosphorylation of Akt in control myotubes after 120 minutes of insulin treatment, and this elevation was blunted in the FoxO1 3A model. Muscle atrophy induced by starvation and glucocorticoid administration has been associated with reductions in Akt signaling and inductions of MAFbx/atrogin-1 activity.[113] Surprisingly, there is evidence to support the role of hyperphosphorylation of Akt upon FoxO1 overexpression in various models of muscle atrophy. Recent work in various cell lines including myotubes, hepatocytes and cardiomyocytes has shown that FoxO1 overexpression results in increased basal phosphorylation of Akt.[26,88,96] Furthermore, Moylan et al[85] reported that Akt phosphorylation was significantly elevated in mouse skeletal muscle cells in vitro in the presence of TNFα, which is a known atrophy inducing molecule. Thus, it appears that in these models, FoxO1 mediated muscle atrophy is occurring despite enhanced Akt signaling. Despite the controversial role of phosphorylation of Akt upon FoxO1 activation, our findings indicated that in relationship to the hearty decrease in protein synthesis, Akt phosphorylation was also blunted upon FoxO1 overexpression and was not rescued after 120 minutes of insulin treatment.

$p70^{s6k}$ has been demonstrated to be responsible for increased translation initiation in response to growth stimulus. Specifically, increased phosphorylation of $p70^{s6k}$ occurs in response to resistance exercise, sprint exercise, and supplementation with amino acids and insulin.[7,63] Thus, increased $p70^{s6k}$ phosphorylation in skeletal muscle after

mechanical loading or with insulin treatment often reflects increased protein synthesis.[51] However, Hornberger et al[52] demonstrated that mechanical load induced hypertrophy elicits increased activity in $p70^{s6k}$, while growth factor induced hypertrophy elicits unaltered activity of $p70^{s6k}$. Further, cell growth is not habitually associated with increased phosphorylation of $p70^{s6k}$, as demonstrated in models of genetic manipulations (e.g. $p70^{s6k}$ knockout) in which cell growth occurs despite lack of $p70^{s6k}$ activation.[94,129] It has also been demonstrated that the impact of either leucine or insulin by itself does not elicit increased $p70^{s6k}$ phosphorylation, despite increased muscle mass.[45] In fact, our data demonstrated that $p70^{s6k}$ activity was unaltered upon FoxO1 overexpression and insulin treatment did not increase activity of $p70^{s6k}$ in either control or FoxO1 3A myotubes. Thus, our current finding suggested that the activity of the purported anabolic $p70^{s6k}$ molecule was not attenuated by FoxO1 overexpression, even when challenged with a growth stimulus.

Evidence demonstrates that phosphorylation of $p70^{s6k}$ leads to downstream phosphorylation of rpS6, thus increased translational efficiency. Phosphorylation of rpS6 is widely associated with increased cell growth both in vivo and in vitro.[82,108] Moreover, rpS6 has been identified to drive translation of 5' TOP mRNAs and protein synthesis. In our model, there was a significant decrease in the phosphorylation of rpS6 in FoxO1 3A myotubes basally and with insulin treatment, when compared to control myotubes. As such, it was our contention that decreased protein synthesis, conferred through FoxO1 overexpression, occurred independently of signaling through $p70^{s6k}$. Rather, the deficiencies in protein synthesis that occurred prior to loss of total protein resulted in

attenuation of phosphorylation of rpS6. Therefore, these data support a deficiency in ribosomal activation upon FoxO1 overexpression.

Importantly, the growth of skeletal muscle in vitro has often been linked to Cyclin D1 expression.[87] Cyclin D1 is a potent regulator of the cell cycle and in order to progress through the cell cycle, Cyclin D1 protein expression increases concomitantly with increases in CDK4 protein.[73] As demonstrated in previous work, the increase in Cyclin D1 protein expression was detected in the nuclei of myotubes, indicating that expression of this cell cycle gene can occur in the multinucleated myotube and is not limited to unfused myoblasts.[58] Additionally, the upregulation of Cyclin D1 has been associated with increased myotube hypertrophy in vitro.[87] In contrast, our data demonstrated no differences in Cyclin D1 protein expression in FoxO1 3A myotubes compared to control myotubes. Additionally, FoxO1 overexpression did not appear to differentially regulate Cyclin D1 activity with insulin treatment, suggesting that alterations in cell cycle regulators was not the causative agent for protein synthesis deficiencies. Rather, suppression of protein synthesis was more likely related to alterations in ribosomal activation, as supported by the rpS6 findings. Though this data suggested that FoxO1 is integral in the suppression of ribosomal activation, we were limited in that only one aspect of translational control was evaluated.

Translation of mRNA is a complex process involving translation initiation, elongation, and termination. In addition, ribosomal biogenesis is a broad term referring to both the translational capacity and translational efficiency of the ribosome. Often times, translational efficiency is determined by phosphorylation or total protein expression of key markers of translation initiation, such as rpS6, 4EBP1, eIF4B, and EF2.

Although FoxO1 3A myotubes suppress protein synthesis in an rpS6 dependent manner, we cannot exclude the possibility that rDNA transcription, rRNA processing, or other activities required for ribosome biogenesis may be affected. As more than 85% of total cellular RNA consists of ribosomal RNA[22,43], the FoxO1 mediated suppression of protein synthesis likely represents a suppression in synthesis of ribosomal proteins. Therefore, it is imperative to further evaluate the role of FoxO1 in translation elongation, termination, and its role in affecting ribosomal translational capacity. Recently, it has been demonstrated that FoxO1 initiates atrophic machinery acutely and sustains muscle atrophy over a long period of time.[110] Our findings demonstrated that the acute activation of FoxO1 initially suppresses protein synthesis; prior to any phenotypic changes in muscle mass. This knowledge expands the understanding of the muscle atrophy program and gives insight into the mechanisms that occur preceding loss of muscle mass or total protein content.

It is imperative to understand the regulation of skeletal muscle growth and loss, particularly in disease states such as Type II Diabetes Mellitus. Skeletal muscle is capable of adapting to various stimuli and these adaptations result in changes in muscle size, metabolism, and function. Specifically, in Type II Diabetes Mellitus, skeletal muscle exhibits increased glycolytic fiber numbers, muscle atrophy and abnormal lipid deposition.[27,54] In fact, it has been demonstrated that the diabetic muscular environment is associated with increased protein degradation and muscle atrophy.[79,83] Interestingly, insulin treatment has been shown to diminish rates of proteolysis in diseased states and lead to increased protein synthesis rates. Therefore, the further understanding of the role of FoxO1 and its impact on protein synthesis mechanisms are essential.

In light of the findings in this study, it is imperative to elucidate the role of FoxO1 in regulating both translational efficiency and translational capacity in order to substantiate the ability of FoxO1 to impact ribosome biogenesis in relation to muscle atrophy. Further research into the mechanisms related to FoxO1 in skeletal muscle may provide breakthrough therapies for the treatment of muscle dysfunction associated with prolonged bedrest, cachexia, and Type II Diabetes Mellitus.

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