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THE EFFECTS OF AGING ON MYOSTATIN PATHWAY ACTIVITY AFTER

THREE SEQUENTIAL BOUTS OF RESISTANCE EXERCISE

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THE EFFECTS OF AGING ON MYOSTATIN PATHWAY ACTIVITY AFTER
THREE SEQUENTIAL BOUTS OF RESISTANCE EXERCISE

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

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"As long as you're working you're not wasting time."

Travis Beck

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ABSTRACT

THE EFFECTS OF AGING ON MARKERS OF MYOSTATIN PATHWAY

ACTIVITY AFTER THREE SEQUENTIAL BOUTS OF RESISTANCE EXERCISE

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Background. Myostatin signaling serves to regulate skeletal muscle mass by influencing genes responsible for regulating satellite cell activity and by participating in glucocorticoid induced skeletal muscle atrophy; however research investigating myostatin pathway signaling is still in its infancy.

Purpose. The purpose of this investigation was two-fold: 1) To examine if baseline differences in myostatin signaling exist between younger (18-35 yr) and older (65-80 yr) men. 2) To examine if short-term, chronic resistance training can ameliorate potential differences in myostatin signaling that exist between younger and older adults.

Methods. Younger (n = 10; age: 21.0 ± 0.5 years, body mass: 82.3 ± 4.2 kg, height: 178.4 ± 2.2 cm, body fat percentage: $15.4 \pm 2.9\%$) and older (n = 10; age: 66.4 ± 1.6 years, body mass: 94.2 ± 3.7 kg, height: 180.9 ± 2.2 cm, body fat percentage: $27.4 \pm 1.8\%$) men chose to participate in the current investigation. Participants were one repetition maximum tested (1 RM) for leg press, hack squat and leg extension. Then participants underwent two familiarization sessions separated by 48 hours before

partaking in three training sessions separated by 48 hours which consisted of 3 sets of 10 repetitions at 80% of 1RM for each of the previously mentioned exercises. Percutaneous muscle biopsies were collected from the *vastus lateralis* prior to the exercise intervention (T1), 48 hours following workout 1 (T2), 48 hours following workout 2 (T3), and 24 hours following workout 3 (T4). The mRNA expression of *MYOSTATIN*, *ACTIVIN IIB*, *HSGT*, *TITIN CAP*, *FLRG*, *FOLLISTATIN* and *SMURF1* were analyzed in duplicate and expressed using the $2^{-\Delta CT}$ method where ΔCT = (gene of interest − the average of *B2M* and *28S*). The protein expression of phosphorylated Smad3 (pSmad3) was determined using western blotting procedures.

Results. Younger men had a significantly greater 1 RM for hack squat (younger: 170.0 ± 10.9 kg, older: 105.8 ± 10.4 kg; p = 0.001), leg press (younger: 271.1 ± 14.6 kg, older: 182.7 ± 14.9 kg; p < 0.001) and leg extension (younger: 64.8 ± 4.0 kg, older: 46.7 ± 2.8 kg; p = 0.002) compared to older men. As a result the cumulative training volume was significantly greater during the training bouts in younger compared to older men (younger: 36,392 ± 1,894 kg, older: 23,724 ± 1,639 kg; p < 0.001). Three day food diaries indicated that relative caloric (young = 33.4 ± 5.1 kcal/kg/d, old = 19.5 ± 1.9 kcal/kg/d; p = 0.038), protein (young = 1.6 ± 0.2 g/kg/d, old = 0.8 ± 0.1 g/kg/d; p = 0.030), carbohydrate (young = 4.1 ± 0.6 g/kg/d, old = 2.3 ± 0.3 g/kg/d; p = 0.017) and fat (young = 1.2 ± 0.2 g/kg/d, old = 0.6 ± 0.1 g/kg/d; p = 0.006) consumption were each significantly greater in younger compared to older men. Non-parametric statistics were used for the assessment of mRNA and protein data. The only between groups differences for the mRNA expression of the genes of interest occurred at baseline (p = 0.038) and T4 (p = 0.005) for *FLRG* and baseline (p = 0.023) and T2 (p = 0.008) for *FOLLISTATIN* in

which older men had significantly greater mRNA expression values compared to younger men. The only significant within group changes occurred in the mRNA expression of *MYOSTATIN* as older men had a significant downregulation following T3 (p = 0.047) and T4 (p = 0.013), while younger men experienced a trend decrease following T4 (p = 0.074). Significant between group differences were present in the protein expression of pSmad3 following T3 (p = 0.012) and T4 (p = 0.010).

Conclusions. Baseline differences in myostatin signaling were present as older men had significantly greater mRNA levels of the myostatin binding proteins *FLRG* and *FOLLISTATIN* compared to younger men. The myostatin pathway signaling response following short-term, chronic resistance training was similar between younger and older men. However, alterations in myostatin pathway signaling following repeated resistance training bouts was more favorable in older men. Specifically, older men experienced a significant decrease in the mRNA expression of *MYOSTATIN* at T3 and T4. Older men also had significantly lower pSmad protein levels at T3 and T4 compared to younger men. Decrements in serum androgen concentrations appear to be primarily responsible for the loss of skeletal muscle mass with age. However, in attempt to maintain skeletal muscle mass with age positive physiological adaptations have been found to occur such as an increase in the mRNA expression of ANDROGEN RECEPTOR and myogenic regulatory factors. Results from the current investigation provide evidence that favorable adaptations occur in myostatin pathway signaling to promote skeletal muscle growth with age and following short-term, chronic resistance training.

CHAPTER I

INTRODUCTION

It is well known that myostatin is an important regulator of skeletal muscle mass but research investigating myostatin pathway signaling is still in its infancy. There are several binding proteins that serve to inhibit the effects of myostatin either by preventing secretion: HSGT[1] and titin cap[2], activation: GASP1[3] and HSGT[1] or receptor binding: myostatin propeptide[4], FLRG[5] and follistatin[6]. Unbound, active myostatin binds to activin IIB receptors which activate the type I receptor (ALK4 or ALK5) which transphosphorylate Smads2/3 which then aggregate with Smad4 and translocate the nucleus and influence gene transcription[7]. The negative regulators of myostatin signaling are Smad7[7] and Smurf1[8]. Smad7 binds to the intracellular domain of type I receptors preventing the phosporalization of Smads2/3[9, 10] and further inhibits myostatin signaling by forming a complex with Smads2/3, thereby reducing the complex formation between Smads2/3 and Smad4[7, 10]. While Smurf1 is an E3 ubiquitin ligase which tags Smad2/3 for degradation[11].

Myostatin has a vital role in the regulation of skeletal muscle mass from the prospective of skeletal muscle hypertrophy and atrophy. Myostatin has been proposed to be a negative regulator of skeletal muscle mass by maintaining satellite cells in a quiescent state[12-14] and is involved with skeletal muscle loss via glucocorticoid induced skeletal muscle atrophy[15, 16]. Animal models have demonstrated the importance of myostatin in the regulation of skeletal muscle mass as male mice over-expressing myostatin have significantly less skeletal muscle mass compared to normal counterparts[17]. While myostatin knockout mice[18, 19] and mice receiving a myostatin blocking anti-body[20, 21] have been found to be significantly stronger and to have

significantly more skeletal muscle mass compared to normal counterparts. Myostatin has also been found to play a role in the regulation of skeletal muscle mass in humans as serum myostatin has been found to be upregulated in accord with muscle wasting in HIV infected men[22].

Due to the importance of myostatin in the regulation of skeletal muscle mass the possibility exists that myostatin pathway signaling may be involved with the age related loss of skeletal muscle mass, but few investigations have directly examined the effects of age on myostatin signaling in humans. In 2002, Roth et al.[23] examined the effects of a 9 week unilateral heavy resistance training program on myostatin gene expression in younger (20-30 yr; 4 men and 4 women) and older (65-75 yr; 3 men and 4 women) adults. The authors found *MYOSTATIN* mRNA expression to be significantly downregulated following chronic resistance exercise (pre: 2.70 ± 0.36; post: 1.69 ± 0.18 arbitrary units) with no significant age or gender differences. However, the results may have been influenced by biopsy time-points as the post-training biopsy was obtained 48-72 hr following the final resistance training bout. *MYOSTATIN* mRNA expression has since been found to be significantly downregulated 48 hours following an acute exercise bout in older adults[24] and no data is currently available on *MYOSTATIN* mRNA expression 72 hours following an exercise bout. In 2006, Raue et al.[25] examined the effects of age and an acute bout of resistance exercise on gene expression in younger (n = 8; 18-30 yr) and older (n = 6; 80-89 yr) women. Muscle biopsies were obtained prior to and 4 hours following resistance exercise. Baseline mRNA expression of *MYOSTATIN* was significantly greater in older compared to younger women. Following training there was a significant downregulation of *MYOSTATIN*

mRNA expression in younger and older women with no significant difference in *MYOSTATIN* mRNA expression between groups. Finally, Jensky et al.[26] examined the effects of an acute resistance exercise bout consisting of single leg eccentric knee extensions on select markers of the myostatin signaling pathway between younger (n = 10; 28 ± 5 yr) and older (n = 10; 68 ± 6) men at baseline and 24 hours following exercise. At baseline there was a trend for *MYOSTATIN* mRNA to be greater in older compared to younger adults (p = 0.06) and older adults had significantly greater levels of *FOLLISTATIN* mRNA expression while no differences between groups were present for *HSGT*. There was no effect of exercise on *MYOSTATIN*, *FOLLISTATIN* or *HSGT* 24 hr post-exercise.

Due to the importance of myostatin in the regulation of skeletal muscle mass through the inhibition of hypertrophy by maintaining satellite cells in a quiescent state[12-14] and by influencing skeletal muscle catabolism through a glucorticoid induced mechanism[15, 16] it is of importance to understand the effects of age on myostatin pathway signaling. Therefore, the purpose of this investigation is two-fold: 1) To examine if baseline differences in myostatin signaling exist between younger (18-35 yr) and older (65-80 yr) men. 2) To examine if short-term chronic resistance training can ameliorate potential differences in myostatin signaling that exist between younger and older adults.

1. It is hypothesized that baseline *MYOSTATIN* mRNA expression will be significantly upregulated in older compared to younger men. However, following resistance training it is hypothesized there will be no significant difference in *MYOSTATIN* mRNA expression between younger and older men.

2. It is hypothesized there will be no significant difference in the baseline mRNA expression *ACTIVIN IIB* receptor at baseline or following resistance training between younger and older men.

3. It is hypothesized there will be no baseline differences between younger and older men for the myostatin binding proteins: *FLRG*, *TITIN CAP* and *HSGT* at baseline or following training. However, it is hypothesized that mRNA expression of *FOLLISTATIN* will be significantly upregulated in older adults compared to younger adults at baseline with no significant differences between groups following training.

4. It is hypothesized that *SMURF1* mRNA expression will be significantly upregulated in older compared to yonger adults at baseline and no significant differences will be present following training.

5. It is hypothesized that pSmad3 protein expression will be significantly greater in older men compared to younger men at baseline and there will be no significant difference between younger and older adults following training.

<u>Definitions of Terms and Procedures</u>

Myostatin – Is a negative regulator of skeletal muscle mass. Myostatin acts to keep satellite cells in a quiescent state and is involved with glucocorticoid induced skeletal muscle atrophy.

Activin IIB – The receptor with the highest binding affinity for myostatin.

R-Smads – Are a class of proteins (Smad1, Smad2, Smad3, Smad5 and Smad8) that when activated bind to the common mediator Smad (Smad4) which translocate the nucleus and regulate the transcription of specific genes. Smads2/3 are activated in response to TGF-β or activin signals and play an importation role in the myostatin signaling pathway for the regulation of skeletal muscle mass Smads1/5/8 are activated in response to bone morphogenetic protein.

Co-Smad – Interact with R-Smads to influence gene transcription. The only known Co-Smad is Smad4.

I-Smads – The known I-Smads are Smad6/7. Smad7 regulates myostatin by preventing the recruitment and activation of Smad2 and 3.

Smurf1 – Is an E3 ubiquitin ligase that tags Smads2/3 for degradation.

HSGT – Is a myostatin binding protein expressed in skeletal muscle which inhibits myostatin secretion and activation.

Titin Cap – Is a myostatin binding protein expressed in skeletal muscle which inhibits myostatin latent complex formation and secretion, maintaining myostatin in an "inactive" state.

Follistatin – Is a myostatin binding protein expressed in skeletal muscle which inhibits myostatin receptor binding.

FLRG also known as FSTL3 - Is the primary binding/inhibition protein of myostatin. Once bound to myostatin FLRG inhibits myostatin receptor binding.

Satellite cells – Skeletal muscle precursor cells that are located between the sarcolemma of mature muscle fibers and the basement membrane.

Quiescence – A term used in reference of satellite cells to describe a state of inactivity; a state in which a cell is not dividing.

Proliferation – A term used in reference of satellite cells to describe a state of activity; a state in which a cell is dividing.

Differentiation – A term used in reference of satellite cells; is the process in which a less specialized cell becomes a more specialized cell.

Cyclin-dependent kinases (CDK) – Are a group of proteins that when upregulated signal satellite cell proliferation.

CDK inhibitors – A term used to refer to proteins (p21, p27, p57, ect.) that when upregulated end satellite cell proliferation allowing satellite cells to start the process of differentiation.

Myogenic regulatory factors (MRF) – Are basic helix-loop-helix proteins (MyoD, myogenin, MRF4 and Myf5) that stop satellite cell proliferation and signal satellite cell differentiation.

Myosin heavy chain – Myosin is one of the contractile components of skeletal muscle, the other being actin. Myosin heavy chain is a protein commonly used to examine the effects of a nutritional/exercise intervention on skeletal muscle hypertrophy.

DNA – Deoxyribonucleic acid is a double-stranded molecule that contains the genetic instructions used in the development and function of living organisms. Genetic

information is transcribed from DNA, located in nuclei, into mRNA and then translated into functional proteins.

Total RNA – Ribonucleic acids are single-stranded molecules. There are three types of RNA:

1) ribosomal RNA (or rRNA including the 18S, 5.8S, 28S and 5S subtypes) make up ~80% of the total RNA pool and are used to carry out protein synthesis; 2) transfer RNA (or tRNA) make up ~15% of the total RNA pool and are used to transfer amino acids to growing polypeptide chains during protein synthesis; 3) messenger RNA (or mRNA) makes up ~5% of the total RNA pool and function to carry genetic information from genes to ribosomes

Muscle homogenation – Refers to the process of using various buffers to make solid muscle soluble in solution for subsequent substrate analyses

Cell lysis buffer – A muscle homogenation buffer that yields total muscle protein solubilized in solution for subsequent western blotting analyses.

Tri reagent – A solution which contains chemicals (i.e., phenol and guanidine thiocyanate) that inhibit RNase activity.

Western blotting – A multi-step process whereby proteins from muscle homogenates are: 1) separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, 2) transferred to a nitrocellulose membrane and 3) probed using a primary antibody specific to a protein of interest. In this process a secondary antibody conjugated to an enzyme which is specific to the primary antibody and a substrate which luminesces when exposed to the secondary antibody-enzyme conjugate emits light which is used to analyze muscle protein content of the specific protein of interest.

Ponceau S staining – Is a staining procedure used to mathematically correct for lane-to-lane loading variations that occur during Western blotting.

Real time reverse transcriptase polymerase chain reaction (RT-PCR) – Is a multi-step procedure which uses gene-specific primer sequences and a fluorescent tracer to detect the expression of specific mRNA transcripts.

Housekeeping gene – Is a gene that is constitutively expressed (in this investigation prior to and following resistance exercise). The housekeeping genes *B2M* and *28S* were used to normalize the gene expression of the genes of interest and to correct for well-to-well loading variations between samples.

Melt Curve – Is a *post hoc* procedure used to confirm the presence of one cDNA amplification product (or gene) during PCR.

Abbreviations

MSTN – myostatin

FLRG - follistatin-related gene, is also known as follistatin-like-3 (FSTL3)

GASP1 – growth and differentiation factor-associated serum protein-1

HSGT - human small glutamine-rich tetratricopeptide repeat-containing protein

Smad - mothers against decapentaplegic homolog

B2M – Beta-2 microglobulin

R-Smads - receptor-regulated Smads

Co-Smad - common-mediator Smad

I-Smads - antagonistic or inhibitory Smads

SMURF1- is also known as SMAD specific E3 ubiquitin protein ligase 1

MURF-1 - muscle-specific RING (really interesting and novel gene) finger

FOXO3A - forkhead transcription factor

PAX3 - paired box 3

PAX7 - paired box 7

KI-67 – antigen identified by monoclonal antibody KI-67 also known as MKI67

P57 - cyclin-dependent kinase inhibitor 1C

CDK – cyclin-dependent kinase

P21^{Cip1} – cyclin-dependent kinase inhibitor 1A

P27^{Kip1} – cyclin-dependent kinase inhibitor 1B

MYOD – myogenic differentiation

MRF4 – myogenic regulatory factor 4 also known as myogenic factor 6 and herculin

MYF5 – myogenic regulatory factor 5

MEF2 – myocyte enhancer factor 2

MHC – myosin heavy chain

MGF – mechano growth factor

RNA – ribonucleic acid

DNA – deoxyribonucleic acid

IGF – insulin-like growth factor

MRF – myogenic regulatory factor

RT-PCR – reverse transcriptase polymerase chain reaction

cDNA – copy DNA

 [ ] – signifies concentration

ALS - amyotrophic lateral sclerosis

PRO – protein

PLA – placebo

Delimitations

Ten older (60-75 yr) and ten younger (18-25 yr) males who were not currently participating in any form of resistance training were recruited for this investigation. Befor any data was collected each participant completed a written statement of informed consent and medical history questionnaire. Inclusion criteria included the following: 1) Participants had to be apparently healthy and could not have participated in a structured resistance exercise regimen (i.e., at least one time per week) for one year prior to the initiation of this investigation. 2) Participants had to abstain from smoking, alcohol, tobacco and caffeine for the duration of the investigation. 3) Participants could not have consumed ergogenic nutritional supplements for at least 3 months prior to the start of the investigation. 4) Participants could not be affected with metabolic disorders including heart disease, arrhythmias, diabetes, thyroid disease or hypogonadism. 5) Participants could not have a history of pulmonary disease, hypertension, hepatorenal disease, clotting disorders, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers or anemia. 6) Participants could not have been diagnosed with a neuromuscular disease that would have prevented them from participating in the resistance training protocol. 7) Participants could not have been taking prescription medications indicated for heart, pulmonary, anti-coagulant, anti-hypertensive, psychotropic, neuromuscular/neurological or androgenic dysfunctions. 8) Participants could not have had an allergy to Lidocaine or latex. 9) Older participants had to provide written medical clearance from their primary physician. 10) Participants could not have had any absolute or relative contraindication for exercise testing as outlined by the American College of Sports Medicine:

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Absolute Contraindications to Exercise Testing

– A recent significant change in the resting ECG suggesting significant ischemia, recent

 myocardial infarction or other acute cardiac event

– Unstable angina

– Uncontrolled cardiac dysrhythmias causing symptoms or hemodynamic compromise

– Symptomatic severe aortic stenosis

– Uncontrolled symptomatic heart failure

– Acute pulmonary embolus or pulmonary infarction

– Acute myocarditis or pericarditis

– Suspected or known dissecting aneurysm

– Acute systemic infection, accompanied by fever, body aches, or swollen lymph glands

Relative Contraindications to Exercise Testing

– Left main coronary stenosis

– Moderate stenotic valvular heart disease

– Electrolyte abnormalities (e.g. hypokalemia, hypomagnesemia)

– Severe arterial hypertension (i.e. SBP > 200 and/or DBP > 110) at rest

– Tachydysrhythmia or bradydysrhythmia

– Hypertrophic cardiomyopathy and other forms of outflow tract obstruction

– Neuromuscular, musculoskeletal, or rheumatoid disorders that are exacerbated by

 exercise

– High-degree atrioventricular block

– Ventricular aneurysm

– Uncontrolled metabolic disease (e.g., diabetes, thyrotoxicosis, or myxedema)

– Chronic infectious disease (e.g., mononucleosis, hepatitis, or AIDS)

– Mental or physical impairment leading to inability to exercise adequately

<u>Assumptions</u>

1. Participants correctly completed the medical history questionnaires.

2. Participants followed the guidelines established by the investigators throughout the duration of the study (i.e., participants refrained from consuming supplements, tobacco, alcohol, anti-inflammatory, etc; participants did not exercise between testing sessions; participants reported to the laboratory prior to each session at least 12 hours fasted).

3. The exercise stimulus employed during the three sequential exercise bouts was an adequate stimulus to elicit favorable physiological responses.

4. The participants recruited in this investigation were representative of younger and older male populations.

5. Participants gave maximal effort during maximal exercise testing and during each resistance training session.

<u>Limitations</u>

Theoretical limitations

1. Younger and older men were recruited from the University of Oklahoma campus and surrounding areas. Furthermore, participants received $150.00 upon completion of the investigation for their time and effort to complete the study. In essence participants were recruited out of convenience rather than random selection.

CHAPTER II

REVIEW OF LITERATURE

Cellular and Hormonal Changes Associated with Sarcopenia

Effect of Age on Skeletal Muscle Morphology

Sarcopenia is an age-dependent loss of skeletal muscle mass resulting in reduced strength, limited mobility and increased injury risk[27]. At the cellular level sarcopenia is characterized by a decrease in skeletal muscle volume[28-31]. In 1983, Lexell et al. examined skeletal muscle fiber characteristics of younger (n = 6, 19-37 yr) and older (n = 6, 70-73 yr) deceased men. Results from the investigation found older men had approximately 110,000 less muscle fibers in the midsection of the vastus lateralis than younger men, resulting in a 23% decline in skeletal muscle fiber content[30]. While Young et al.[28, 29] used ultrasound imaging to find the cross-sectional area of the quadrecepts muscle in older men and women (70-80 yr) to be 25-35% smaller than younger men and women (20-30 yr). In this regard a majority of investigations examining muscle morphology of the vastus lateralis have found type II skeletal muscle fiber diameter is reduced to a much larger extent than type I skeletal muscle fibers with age[31-37].

The loss of skeletal muscle mass with age is well described[28-31], but results regarding skeletal muscle fiber distribution with age have been equivocal. In 1978, Larsson et al.[34] found type I fibers to compose 39% of the skeletal muscle fibers in the vastus lateralis of 20-29 yr old men while type I fibers composed 66% of skeletal muscle in 60-65 yr old men. However, a review of research suggests the vastus lateralis of young muscle to be composed of approximately 50% of type I fibers[32] suggesting the

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percentage of type I fibers in the investigation by Larsson et al.[34] may have been underestimated in young adults. In support of this theory Lexell et al.[38] did not find a significant difference in the proportion of type I skeletal muscle fibers in the vastus lateralis between 24 (n = 10), 52 (n = 6) and 77 (n = 8) yr old men. Moreover, Frontera et al. found type I skeletal muscle fiber content to decrease from 60% to 42% in the same group of subjects from the age of 65 ± 4.2 yr to 77 ± 4.2 yr. The equivocal results regarding the effects of age on type II skeletal muscle fiber prevalence may be explained by sampling variability, as the proportion of fiber types has been found to vary depending on the depth of the muscle sample obtained from the vastus lateralis[31, 39]. As a result it has been suggested that the overall reduction in skeletal muscle fiber number with age seems to effect type I and type II fibers to the same extent[32, 40]. Neurological factors also play a role in sarcopenia as there is a loss of functional motor units with age[41, 42]. As a result skeletal muscle fibers experience states of denervation and reinnervation with age[43], which causes a grouping of type I and type II fibers in older adults rather than the random distribution of skeletal muscle fibers typically found in younger adults.

Hormonal Adaptations with Age

Hormones are responsible for regulating skeletal muscle adaptations by priming the body for muscle protein synthesis with the secretion of anabolic hormones or muscle protein catabolism with the secretion of catabolic hormones[44]. With age serum concentrations of anabolic hormones and growth factors have been found to decline[45-50] and reductions in anabolic hormone concentrations have been found to correlate with the age related declines in skeletal muscle mass and strength[51, 52]. Research on the effects of age on catabolic hormones is limited but serum cortisol concentrations have

been found to be significantly linearly correlation with age in women[53], but not men[46, 53].

There are also differences present between younger and older adults in regard to the hormonal response following exercise[54-56]. Kraemer et al.[46] examined the effects of chronic (10 week) heavy resistance training in physically active younger (29.8 ± 5.3 yr) and older (62.0 ± 3.2 yr) men. Serum was obtained before, immediately after and 5, 15 and 30 minutes post-exercise prior to initiation of the exercise program and following 3, 6 and 10 weeks of training. At baseline there were no significant differences between younger and older men for total testosterone, free testosterone, growth hormone or cortisol. There were significant differences between younger and older men for IGF-1 and IGFBP-3. During the resistance training session younger men experienced a more robust increase in total testosterone, free testosterone and growth hormone than the older men. Furthermore, following the 10 week training program younger adults experienced a significant increase in resting free testosterone and IGFBP-3, of which each was significantly greater than the resting level of older men. Finally, following chronic resistance training younger men had lower serum concentrations of adrenocorticotropic hormone (ACTH) and cortisol compared to older men. Taken together these results suggest younger adults create a superior anabolic environment compared to older men allowing for greater increases in skeletal muscle strength and hypertrophy immediately following an acute resistance training bout at rest and following chronic resistance training. Roberts et al.[45] also found serum free testosterone concentrations to decrease with age; however, androgen receptor concentrations were found to be significantly upregulated in the skeletal muscle of older (n = 13, 67.6 ± 1.3 yr) compared to younger (n

= 11, 21.3 ± 0.6 yr) men. Given these findings androgen receptor concentrations may increase to counterbalance the lower levels of free testosterone in an effort to preserve skeletal muscle mass with age.

Decrements in Protein Synthesis with Age

There is a significant decrease in the rate of muscle protein synthesis between younger and older adults[57-59]. In 1993, Yarasheski et al.[57] determined the rate of quadriceps muscle protein synthesis using intravenously infused [^{13}C] leucine to assess the rate of mixed muscle protein synthesis in younger (n = 6, 24 yr) and older (n = 6, 63-66 yr) men and women before and following 2 weeks of daily heavy resistance training. Prior to training the fractional rate of muscle protein synthesis (percent muscle mass of quadriceps/hr) was significantly higher in younger compared to older adults. Moreover, following training the rate of muscle protein synthesis significantly increased in both groups and a significant difference was no longer present between younger and older adults. However, the finding that mixed muscle protein synthesis was increased calls for further investigation as non-functional components of muscle could have been increased to a greater extent in older compared to younger adults (i.e. collagen synthesis rate). In 1995, Welle et al.[59] determined myofibrillar protein synthesis rates using tracer L-[1-13C]leucine into myofibrillar proteins obtained from the vastus lateralis muscle by needle biopsy in younger (n = 9, 22-31 yr) and older (n = 9, 62-72 yr) men and women at baseline and following 3 months of a progressive resistance training program. Prior to exercise the mean fractional myofibrillar synthesis rate was significantly slower in older compared to younger adults. Following training the fractional myofibrillar synthesis rate non-significantly increased in each group and the younger adults maintained a

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significantly higher myofibrullar protein synthesis rate compared to older adults. Suggesting younger adults maintain significantly higher rates of functional muscle protein synthesis compared to older adults following chronic resistance training.

Gene transcription must occur prior to translation and thus protein synthesis. A primary protein of interest in regard to skeletal muscle adaptations following resistance training is myosin heavy chain (MHC). As a result Balagopal et al.[58] examined the effects of age on mRNA expression of the *MHC* isoforms (*MHCI, MHCIIA* and *MHCIIX*) in younger (n = 7, 20-27 yr), middle aged (n = 12, 47-60 yr) and older (n = 14, > 65 yr) men. Baseline testing revealed a trend decrease for *MHCI* mRNA expression with increasing age although no significant differences were present between the groups. However, there was a significant decrease in mRNA expression of *MCHIIA* and *MHCIIX* from younger to middle age and from middle age to older participants. The authors also examined the effects of age and chronic resistance training on the transcript levels of the *MHC* isoforms and the fractional synthesis rate of *MHC* in 39 participants ranging in age from 46-79 yr. Exercise was found to significantly increase the fractional synthesis rate of mixed muscle protein and *MHC*. Further analyses revealed exercise training to significantly increase mRNA expression of *MHCI* and significantly decrease *MHCIIA* and *MHCIIX* isoforms. However, numerous investigations have found the diameter of type II fibers to increase in older adults following chronic resistance training[60-64].

Effect of Age on Satellite Cells

A primary adaptation of aging is a loss of skeletal muscle mass with type II fibers experiencing a greater loss of volume than type I fibers[31-37]. The fiber specific decrease in type II skeletal muscle volume may be related to a fiber specific decrease in

the number of satellite cells, which are the only source for the generation of new myonuclei *in vivo* in skeletal muscle tissue[65-67] and as a result are responsible for the maintenance of skeletal muscle mass[68]. To test this hypothesis Verdijk et al.[68] examined fiber specific adaptations in the prevalence of type I and type II skeletal muscle fibers with age in younger (n = 8, 20 ± 1yr) and older (n = 8, 76 ± 1 yr) men. Data analyses revealed that older men had significantly less satellite cells per type II skeletal muscle fiber compared to younger men. Additionally, older men had less satellite cells per type II muscle fiber than type I muscle fiber, suggesting the loss of satellite cells with age is fiber specific.

In a follow-up study Verdijk et al.[69] examined the effects of chronic resistance training on fiber specific hypertrophy and adaptations in satellite cell content in older men (n = 13, 72 ± 2 yr). Muscle biopsies were collected from the vastus lateralis 3 days prior to the onset of training and 4 days following the final resistance training session. The training regime was effective at significantly enhancing leg strength, leg lean mass and quadriceps cross-sectional area. At baseline mean fiber area and satellite cell content were smaller in the type II compared to type I fibers. The training regime resulted in significant increases in type II fiber area and satellite cell content while there were no significant changes in the size or satellite cell content in type I fibers suggesting physical activity may ameliorate the decrease in fiber specific satellite cell content with age. An earlier study conducted by Roth et al.[70] examined satellite cell adaptations following 9 weeks of unilateral lower body resistance training in younger and older men (younger: n = 7, 25 ± 3 yr; older: n = 7, 69 ± 3 yr) and women (younger: n = 7, 26 ± 1 yr; older: n = 7, 67 ± 3 yr). Satellite cell content and the number of active satellite cells were found to

significantly increase following chronic resistance training. Taken together chronic resistance training appears to increase the total number of satellite cells, which may reflect a fiber specific increase of satellite cells in type II skeletal muscle fibers in older adults. Chronic resistance training also increases the number of active satellite cells in younger and older adults.

Effects of Age on Strength and Hypertrophy Adaptations in Response to Chronic Resistance Training

Research has clearly established older adults who engage in a progressive resistance training regimen will obtain significant increases in skeletal muscle strength[60, 62-64, 71-74] and hypertrophy[60, 62-64, 71-75] (the mean age for participants in each referenced investigation was > 60 yr). For an extensive review of typical increases in strength and hypertrophy in older adults following chronic resistance training see Porter et al.[32]. Even though older adults experience increases in strength and hypertrophy following chronic resistance training there may be a differential response between younger and older adults following chronic training in regard to skeletal muscle hypertrophy. In 1999, Kraemer et al.[46] had younger (29.8 ± 5.3 yr) and older (62.0 ± 3.2 yr) men perform a 10 week progressive resistance training program characterized by 3 training sessions per week. At baseline and following chronic resistance training the experimenters assessed 1 RM squat and total thigh muscle cross-sectional area using MRI analysis. Following chronic resistance training there was a significant increase in the delta change ((post value – pre value) / pre value) for 1 RM squat in younger and older men with no significant difference present between groups.

Younger and older men also experienced a significant increase in the delta change for total thigh muscle cross-sectional area, but younger men experienced a significantly greater increase in hypertrophy following training than older men. Results from the current investigation suggest resistance training to be beneficial in terms of muscle strength and hypertrophy adaptations in younger and older men; however, physiological adaptations that occur with aging appear to blunt the hypertrophic adaptations in older men.

Effects of Myostatin on Bone Strength and Morphology

The positive relationship between skeletal muscle mass and bone mineral density (BMD) in humans has clearly been established[76-79]. However, correlations between skeletal muscle mass and BMD may be due to extraneous variables. For example, activity levels have the ability to influence bone formation by altering the load placed on bones and/or by increasing circulating levels of growth hormone[80]. Until recently scientists have been unable to directly examine the effects of skeletal muscle mass on bone morphology and strength, as people and animals with greater skeletal muscle mass are likely to be more active than their counterparts with less skeletal muscle mass. Myostatin null mice have allowed scientists to examine the effects of skeletal muscle mass on bone strength and morphology while controlling for physical activity, as myostatin null mice tend to have 40-100% more muscle mass compared to normal mice[81].

In 2000, Hamerick et al.[82] used adult wild-type hybrid mice and myostatin null mice (type: 129/SvJ/C57BL/6J) who were age and weight matched to examine the effects of muscle fiber hypertrophy and hyperplasia on bone shape and cross-sectional geometry.

Myostatin null mice were found to have significantly larger third trochanters than the wild-type mice, but there were no significant differences between the myostatin null and wild-type mice for cortical area, bending moment of inertia and polar moment of inertia. In 2002, Hamerick et al.[83] examined the effects of increased muscle mass on bone morphology by examining the bone mineral content (BMC) and BMD in the humeri of myostatin null mice (type: 129/SvJ/C57BL/6J) and wild-type hybrid mice. Myostatin null mice weighed significantly more than the wild-type mice and had significantly larger triceps and deltoids. Moreover, the myostatin null mice had a significantly greater trabecular area and trabecular BMC in the proximal humerous (15% length) and significantly greater BMC cortical area, and periosteal circumference in the deltoid crest (40% length). Results from these studies suggests[82, 83] the increased muscle mass of myostatin null mice primarily effects bone at the sites of skeletal muscle insertion, but does not appear to increase diaphyseal strength or axial rigidity. This finding should be expected as activin IIB receptors are not expressed at significant levels in bone[84]; therefore, any effect myostatin has on bone is indirect and primarily influenced through skeletal muscle mass.

Effects of Myostatin on Body Fat and Hyperglycemia

Myostatin has gained popularity in the scientific community primarily due to the effects of myostatin inhibition on skeletal muscle function and morphology. However, myostatin is also gaining popularity as a mechanism to combat the obesity[85-87] and type II diabetes pandemics[85, 86]. In 2002, McPherron and Lee[85] examined the effects of body fat accumulation and hyperglycemia in myostatin-deficient mice (type:

C57BL/6J) compared with wild-type littermates. There were no differences in fat pad weight of myostatin null (MSTN$^{-/-}$) and myostatin expressing (MSTN$^{+/+}$) mice at 2 months of age, but by 9-10 months of age MSTN$^{-/-}$ had 70% less total body fat compared with MSTN$^{+/+}$ mice. Further examination revealed that MSTN$^{-/-}$ mice had 25% fewer gonadal fat pad cells than MSTN$^{+/+}$ mice, reflecting a significant difference in fat cell number. Fat cell size was also significantly different between groups as the mean weight of cells in the genital fat pad was 40% lower MSTN$^{-/-}$ compared to MSTN$^{+/+}$ mice. Also of interest were the findings that MSTN$^{-/-}$ mice consumed significantly more food, had a higher absolute resting VO$_2$ and had less brown adipose tissue than MSTN$^{+/+}$ mice. There were no differences between MSTN$^{-/-}$ and MSTN$^{+/+}$ mice for respiratory exchange ratio. Furthermore, MSTN$^{-/-}$ mice were found to have significantly lower serum levels of leptin, triglycerides and cholesterol than MSTN$^{+/+}$ mice.

Since the absence of myostatin in healthy mice was able to prevent the accumulation of body fat with age and preserve health McPherron and Lee[85] analyzed the effect of the myostatin mutation in two genetic models of obesity, agouti lethal yellow (A^y/a) and obese (Lep$^{ob/ob}$) mice. A^y is a mutation that causes obesity by increasing food consumption and fuel efficiency[88, 89]; whereas, Lep$^{ob/ob}$ causes obesity as the result of the loss of leptin signaling resulting in the improper regulation of food consumption and energy expenditure[90, 91]. Fat pad weights were significantly greater in the A^y/a, MSTN$^{-/-}$ mice compared to the MSTN$^{-/-}$ mice, but weighed significantly less than A^y/a, MSTN$^{+/+}$ mice, suggesting myostatin partially suppressed fat accumulation in A^y mice. Moreover, A^y mice have been used as a model for type 2 diabetes as they have been found to develop insulin resistance[88, 89]. A^y/a, MSTN$^{-/-}$ mice had non-

significantly lower fasting blood glucose concentrations compared to A^y/a, MSTN$^{+/+}$ mice; however, A^y/a, MSTN$^{-/-}$ mice had significantly lower blood glucose levels following an exogenous glucose load compared to A^y/a, MSTN$^{+/+}$ mice. The myostatin mutation was also effective at reducing the fat weight of mice at 8 weeks of age as the Lep$^{ob/ob}$, MSTN$^{-/-}$ mice had significantly less retroperitoneal and parametrial fat pad weights than the Lep$^{ob/ob}$, MSTN$^{+/+}$ mice. Results from this investigation suggest the myostatin mutation has positive effects on obesity and glucose tolerance in healthy and disease prone populations.

A later study by Zhao et al.[86] examined the effects of body fat accumulation in wild-type compared to myostatin null mice fed varying diets. All mice were weaned at 4 weeks of age and were given free access to a normal fat diet (10% of kcals from fat) until 9 weeks of age. Mice from each genotype were then randomly assigned to consume an *ad libitum* normal (low) fat diet (10% of kcals from fat) or an *ad libitum* high fat diet (40% of kcals from fat) until week 18 of life. The high fat diet resulted in the accumulation of 170-214% more fat mass in subcutaneous, epididymal and retroperitoneal fat in wild-type compared to myostatin null mice. Wild-type mice consuming a high fat diet had significantly greater fasting blood glucose, insulin, leptin and resistin (resistin is an adipocyte hormone that potentially links obesity to diabetes as consumption of fat increases resistin, which may cause insulin resistance in skeletal muscles[92]) concentrations compared to the other groups. While serum leptin and adiponectin (adiponectin can directly increase fatty-acid transport, oxidation and dissipation in skeletal muscle resulting in decreased levels of intramyocellular lipids and improved insulin sensitivity in muscle cells and hepatocytes[93]) concentrations were significantly

lower in myostatin null mice consuming a low fat diet compared to the other groups. Results from this investigation provide further evidence suggesting the myostatin mutation provides protection against obesity and may help prevent hyperglycemia, particularly in individuals consuming a high fat diet.

It has been established that myostatin gene knockout causes a significant increase in myogenesis and a significant decrease in adipogenesis[85, 86, 94], while transgenic mice that overexpress myostatin selectively in skeletal muscle have been found to have less muscle mass and more fat mass compared to wild-type mice[17]. As a result Milan et al. [87] examined the effects of weight loss induced by biliopancreatic diversion (BPD) on skeletal muscle myostatin expression. Subjects consisted of 6 morbidly obese (BMI $\geq$ 40 kg/m^2) subjects with normal glucose tolerance and were free from endocrine and non-endocrine diseases. Muscle biopsies were obtained at baseline and following 18 ± 2 months after the BPD operation. All post-biopsies were obtained when subjects reached a weight stable condition. There were significant differences in body weight, fat mass, fat free mass and *MYOSTATIN* mRNA expression quantified using real-time polymerase chain reaction (RT-PCR). Reductions in *MYOSTATIN* mRNA expression were significantly correlated with the reduction in fat free mass ($r = 0.83$; $p < 0.05$). *MYOSTATIN* mRNA expression may have been reduced in an effort to preserve skeletal muscle mass, as subjects in this study lost an average of 42.44 ± 8.35 pounds of fat mass ($p = 0.009$) and 4.31 ± 3.13 kg of fat free mass ($p = 0.03$). Results from the current investigation suggest myostatin may function as a regulator of skeletal muscle mass during periods of caloric restriction that result in rapid weight loss.

Effect of Myostatin on Skeletal Muscle Mass

Myostatin is a protein that limits skeletal muscle growth[95] and as a result myostatin null mice have been found to have increased skeletal muscle mass[18, 19, 96] muscle growth in the form of hypertrophy[18, 19, 96] and hyperplasia[97], a shift in muscle fiber type in which a greater portion of type IIb fibers are present[20] quicker rates of muscle recovery following injury assessed by the size of skeletal muscle fiber diameter following injury[19, 20] and reduced body fat[85-87]. The effects on myostatin on skeletal muscle have been examined via myostatin gene knockout[18, 19, 21, 98], administration of a myostatin-blocking antibody[21] and the genetic over-expression of myostatin[17]. As a result this section will describe the effects of myostatin on skeletal muscle mass.

In 2003, Whittemore et al.[21] examined the effects of an inhibitory antibody (JA16) of myostatin on skeletal muscle mass and health parameters in adult male and female mice ranging in age from 5 to 24 weeks. Myostatin inhibition had no effect on clinical serum safety markers, epididymal fat pad, inguinal fat pad, kidney, liver, or heart weight or histology. However, myostatin inhibition was found to increase grip strength and skeletal muscle mass from 13-30% after 2-4 weeks of treatment, resulting in a significant effect. In 2006, Welle et al.[98] compared the myofibrillar protein synthesis rates and muscle mass of myostatin deficient mice ($MSTN^{\Delta E3/\Delta E3}$) and mice with normal myostatin expression ($MSTN^{+/+}$) between the ages of 5-6 weeks and 6 months of age. At 5-6 weeks of age $MSTN^{\Delta E3/\Delta E3}$ mice had significantly more muscle mass (40%) and a greater rate of muscle protein synthesis assessed by the fractional rate of myofibrillar synthesis (14%) and protein synthesis per whole muscle (60%) in comparison to

MSTN$^{+/+}$ mice. At 6 months of age MSTN$^{\Delta E3/\Delta E3}$ mice still had significantly more muscle

mass (90%) and myofibrillar synthesis per muscle (85%) relative to MSTN$^{+/+}$ mice, but

there were no differences between groups in regard to the fractional synthesis rate

between groups. The synthesis rate per whole muscle is a better indicator than the

factional synthesis rate in terms of determining skeletal muscle size[98]. However, the

increase in protein synthesis per whole muscle does not completely explain the increased

skeletal muscle size in MSTN$^{\Delta E3/\Delta E3}$ compared to MSTN$^{+/+}$ mice. Previous investigations

have found DNA content per muscle to increase approximately 50% in myostatin null

mice[81, 98], but the increase in protein synthesis rate in the current investigation was

found to be approximately 85%[98]. As a result the synthesis rate per myonuclei is

increased in myostatin deficient mice suggesting skeletal muscle hypertrophy may be

more complex than myoblast fusing to an existing muscle fiber to increase RNA

production and protein synthesis, as RNA and mRNA concentrations are not significantly

different in myostatin null and normal mice, while the amount of DNA per mg of muscle

is significantly reduced in MSTN$^{\Delta E3/\Delta E3}$ compared to MSTN$^{+/+}$ mice mice[98].

Myostatin may also influence skeletal muscle mass by regulating glucocorticoid

induced skeletal muscle atrophy[15, 16]. Previous investigations have found

dexamethasone (a glucortciod) to increase endogenous myostatin transcription in C_2C_{12}

myocytes due to a glucorticiod receptor mediated mechanism and the response was found

to occur in a dose-dependent manner[99, 100]. In a follow-up investigation Ma et al.[15]

examined the effects of dexamethasone on *MYOSTATIN* mRNA and protein expression

as well as muscle atrophy in 60, 10-12 week old male Sprague-Dawley rats. The rats

were randomly assigned to one of six groups. Rats in the dexamethasone group received

daily administration of 60, 600, or 1,200 µg/kg body weight for a period of 5 days while

rats in the placebo group received an equivalent amount of saline solution. There was a

significant dose-dependent loss of body weight (-4.0, -13.4 and -17.2% respectively) and

muscle atrophy (6.3, 15.0 and 16.6% respectively) with a resulting significant increase in

MYOSTATIN mRNA (66.3, 450.0 and 527.6% respectively) and protein expression (0.0,

260.5 and 318.4% respectively) in rats receiving dexamethasone compared to controls.

As a result the authors concluded that muscle loss induced by dexamethasone is at least

partially attributed to increased myostatin expression through a glucocorticoid receptor

mediated pathway.

Since myostatin has been found to be significantly upregulated by glucocorticoids

(dexamethasone)[15, 99, 100] Gilson et al.[16] examined if myostatin knockout could

prevent skeletal muscle atrophy resulting from the presence of glucocorticoids. A total of

28 male wild-type myostatin null (MSTN$^{-/-}$) and 28 normal male wild-type mice

(MSTN$^{+/+}$) were randomly assigned to receive dexamethasone at a dose of 1 mg/kg of

body weight per day for 10 days (low dose) or 5 mg/kg of body weight for 4 days (high

dose). Following low dose dexamethasone administration MSTN$^{+/+}$ mice experienced a

significant downregulation in muscle mass and muscle fiber cross-sectional area (CSA)

while muscle mass and muscle fiber CSA were unaffected in MSTN$^{-/-}$ mice following

dexamethasone administration. Following high dose dexamethasone administration

MSTN$^{+/+}$ mice had a significant upregulation of ATROGIN1, MURF1, CATHEPSIN L

and *FOXO3A* while there was no significant effect on the mRNA expression of these

proteolytic genes in MSTN$^{-/-}$ mice. As a result this investigation provides strong evidence

that myostatin plays a critical role in mediating skeletal muscle proteolysis driven by glucocorticoids.

Animal models of myostatin knockout[18, 19, 96], myostatin inhibition with a myostatin-blocking antibody[21] and myostatin over-expression (in male mice only)[17] have found myostatin to be a key factor in regulating skeletal muscle mass. Furthermore, in diseased humans[22] an increase in the presence of myostatin occurs concurrently with the loss in skeletal muscle mass. However, in healthy humans there appears to be no correlation between *MYOSTATIN* mRNA expression and myofribullar protein synthesis[101], strength[23] or muscle mass[23, 101]. However, serum myostatin has been found to be corrected with muscle mass corrected for height (mass/height^2)[102, 103]. In 2007, Kim et al.[104] had 66 participants including younger (20-35 yr, n = 37) and older (60-75 yr, n = 29) men and women participate in a 16 week resistance training program. Muscle biopsies were obtained at baseline and 24 hours following the first and final resistance training bout. Based on mean muscle fiber hypertrophy participants were classified as extreme (n = 17, 2,475 μm^2), modest (n = 32, 1,111 μm^2) or non-responders (n = 17, -16 μm^2) and compared for statistical analyses. Of interest were the findings that *MYOSTATIN* mRNA expression was significantly downregulated 24 hours following the first and final training bout in each group, with no statistical difference between groups. Protein expression of the complete myostatin complex was significantly upregulated following 16 weeks of training with no differences present between groups. While protein expression of the myostatin propeptide were not significantly affected by training and there were no significant differences present between groups. In this investigation and others serum myostatin has been found to be highly variable between

participants[103-106]; nevertheless, serum myostatin was not affected by training and no significant differences between groups were present at any time-point. Also of note was the finding that there was no significant effect of training or group on *ACTIVIN IIB* receptor mRNA expression at any time-point[104]. Overall, results from the current investigation highlight the differential response among healthy participants to a standard resistance training protocol. Furthermore, myostatin is apparently not entirely responsible for the differential response in skeletal muscle hypertrophy following chronic resistance training.

Effect of Exercise on Myostatin

Myostatin Pathway Response Following an Acute Bout of Exercise

Many hormones are influenced by diet and or exercise which stimulate the muscle modulating response experienced with training. For instance, resistance training has been found to increase the secretion of cortisol which is responsible for skeletal muscle atrophy while also increasing the concentrations of anabolic hormones responsible for the stimulation of skeletal muscle hypertrophy such as testosterone and IGF-1. Myostatin has been found to be influenced by aerobic and anaerobic exercise in animal and human models. Louis et al.[107] examined the expression of *MYOSTATIN* mRNA following a 30 minute run at 75% of VO_2 max or resistance exercise consisting of 3 sets of 10 repetitions at 70% of 1 RM knee extensions. *MYOSTATIN* mRNA concentrations were analyzed from muscle biopsies obtained from the vastus lateralus at baseline, immediate post exercise (0 hr), 1, 2, 4, 8, 12 and 24 hr post-exercise. Aerobic and resistance exercise resulted in the downregulation of *MYOSTATIN* mRNA concentrations at each time-point.

Specifically *MYOSTATIN* mRNA expression was significantly decreased at 8 and 12 hr following aerobic exercise and at 1, 2, 4, 8, 12 and 24 hr following resistance exercise. Additional genes of interest were assessed and will be discussed in terms of the response following resistance exercise. First, the mRNA expression of *TNF-α* was significantly upregulated immediately following, 2, 4, 8, 12 and 24 hr following resistance training. Ubiquitin proteasome pathway activity was assessed by examining the mRNA expression of *MURF-1*, *ATROGIN-1* and *FOXO3A*. *MURF-1* was found to be significantly upregulated 1, 2 and 4 hr post-exercise. *ATROGIN-1* was significantly downregulated 8 and 12 hr post-exercise, while there was a trend decrease in the expression of *FOXO3A* 8 and 12 hrs post-exercise ($p \leq 0.07$).

In 2006, Raue et al.[25] examined the effects of age and an acute bout of resistance exercise on myogenic gene expression in younger (n = 8; 18-30 yr) and older (n = 6; 80-89 yr) women. Training consisted of three sets of ten repetitions at 70% of 1 RM on a bilateral knee extension machine. Muscle biopsies were obtained prior to and 4 hours following resistance exercise. Myogenic gene expression was determined by examining the mRNA expression of *MYOSTATIN* and the muscle regulatory factors (MRFs), specifically, *MYOD*, *MRF4*, *MYF5*, *MYOCYTE ENHANCER FACTOR 2* (*MEF2*) and *MYOGENIN*. At baseline *MYOSTATIN*, *MYF5*, *MYOD*, *MYOGENIN* and *MRF4* mRNA expression were significantly greater in older compared to younger women. Following resistance exercise *MYOSTATIN* mRNA expression was significantly downregulated in younger and older women and there was a non-significant downregulation of *MYF5* in younger and older women. There was a significant increase in *MYOD* and *MRF4* following resistance training in younger and older women. While

MYOGENIN was non-significantly upregualted in older and younger women following resistance exercise. Muscle regulatory factors have been found to be upregulated with age[108-111] and the authors concluded the upregulation of *MYF5*, *MYOD*, *MYOGENIN* and *MRF4* mRNA expression in older compared to younger women at rest may be a mechanism to preserve skeletal muscle mass with age. Since older adults have higher mRNA levels of MRFs one would expect more skeletal muscle growth at rest in older compared to younger adults. However, it has been established there is not a 1:1 ratio between mRNA and protein expression as older (30 months) Brown Norway rats have been found to express significantly higher mRNA levels of MRFs but have lower levels of MRF protein compared to younger (9 months) rats[112].

In 2008 Hulmi et al.[24] examined the acute effects of protein ingestion and resistance exercise on myogenic gene expression in older adults. Participants were randomly assigned to a protein group (n = 9; 61.4 ± 4.3 yr) or placebo group (n = 9; 62.1 ± 4.2 yr). Participants in the protein group consumed 15 g of whey protein isolate while participants in the placebo group consumed an equivalent amount of a non-energenic placebo prior to and immediately following the resistance training session. Biopsies were obtained at baseline, 1 hr and 48 hr following an exercise bout consisting of 5 sets of 10 repetitions on a leg press with 2 minute rest periods between sets. The placebo group experienced a non-significant decrease in *MYOSTATIN* mRNA expression 1 hr post-exercise, but had a significant decrease in *MYOSTATIN* mRNA expression 48 hr post-exercise. The protein group experienced a non-significant decrease in *MYOSTATIN* mRNA 1 hr post-exercise and a non-significant increase in *MYOSTATIN* mRNA expression 48 hr post-exercise. *FLRG* mRNA expression was non-significantly

upregulated in the placebo group at 1 and 48 hr post-exercise and the protein group at 1 hr post-exercise; however, *FLRG* mRNA expression was significantly upregulated in the protein group 48 hr post-exercise. *ACTIVIN IIB* receptor mRNA expression was non-significantly decreased 1 and 48 hr post-exercise in the placebo group and non-significantly increased 1 and 48 hr post-exercise in the protein group. *MYOGENIN* mRNA expression was unaffected 1 hr post-exercise and non-significantly upregulated 48 hr post-exercise in the placebo and protein groups. *MYOD* and *P27* were non-significantly upregulated at 1 and 48 hr post-exercise in the placebo and protein groups. While *P21* and *CDK2* mRNA expression were upregulated at each time point in the placebo and protein groups with a significant upregulation in *P21* occurring in the placebo group 48 hr post-exercise and a significant upregulation in *CDK2* occurring in the protein group at 48 hr post-exercise[113].

As expected *MYOSTATIN* mRNA expression is downregulated following an acute bout of resistance exercise[24, 25, 107, 114]. In theory the downregulation of myostatin allows for satellite cell activation resulting in an increase in skeletal muscle mass. For skeletal muscle growth to occur satellite cells must proliferate and then differentiate. As a result mRNA expression of *MYOSTATIN* should decrease following resistance exercise to activate satellite cells. Then *P21* should upregulate leading to the downregulation of *CDK* expression allowing for the upregulation of MRFs which signal satellite cell differentiation and thus skeletal muscle hypertrophy. However, the mRNA expression of genes regulating satellite cell activity following resistance exercise have not been consistent in the literature. The typical time course response for *MYOSTATIN* and

prominent satellite cell regulatory factors following an acute resistance training exercise bout can be seen in Table 1.

Myostatin Pathway Response Following Chronic Resistance Training

One of the first studies to examine the effects of chronic resistance training on *MYOSTATIN* mRNA expression was conducted by Roth et al.[23]. A total of 8 younger (20-30 yr) and 7 older (65-75 yr) sedentary men and women participated in a 9 week unilateral, progressive, heavy resistance training program. All participants trained 3 days per week for 9 weeks. During each training session participants completed 50 near maximal leg extensions on a pneumatic resistance machine with 90-180 second rest periods between sets. Measures of 1 RM strength, percent body fat (using DEXA), thigh muscle volume (using MRI) and *MYOSTATIN* mRNA expression were assessed 2 weeks prior to strength training and 48-72 hrs following the final strength training session. *MYOSTATIN* mRNA expression significantly decreased following chronic resistance training and no gender or age differences were noted for *MYOSTATIN* mRNA expression. Also of interest was the finding that participants with lower baseline levels of *MYOSTATIN* mRNA were more likely to experience little to no change in *MYOSTATIN* mRNA expression following chronic resistance training. Furthermore, no significant correlations were found for baseline *MYOSTATIN* mRNA levels and body mass, muscle strength or muscle volume which is consistent with animal models[115, 116].

In 2004, Walker et al.[117] examined the effects of a whole body resistance training program (n = 11) verse training of the elbow flexors (n = 6) in men (18-45 yr). Training consisted of 2 resistance training session per week for 10 weeks. Pre and post

measures of strength (1 RM elbow flexion), muscle endurance (maximal number of repetitions performed at 80% of 1 RM elbow flexion strength), muscle cross sectional area (MRI), resting levels of plasma IGF-1 (RIA analysis) and plasma myostatin (Western blotting) were obtained. Each resistance training program resulted in significant improvements in elbow flexion 1 RM strength, muscle endurance and elbow flexor muscle CSA. Training was found have no significant effect on plasma IGF-1 concentrations, but a significant decrease was found for plasma myostatin following training in both groups. Individual changes for plasma myostatin ranged from +5.9 to -56.9% resulting in a mean decrease of $20 \pm 16\%$. Results from the present investigation suggest that resting plasma myostatin concentrations may be a more important regulator of skeletal muscle mass than resting plasma IGF-1 concentrations. However, an earlier investigation examining the mRNA expression of *IGF-1* and *MYOSTATIN* between younger (n =12; 21-31 yr) and older (n = 15; 62-77 yr) adults suggest a combination of factors is most likely responsible for sarcopenia. Specifically, the mean *IGF-1* mRNA concentration (mRNA *IGF-1* pre ng total RNA) in older adults was significantly lower (~25%) compared to younger adults, while no significant difference was present in mean *MYOSTATIN* mRNA concentrations between younger and older adults. Finally, no significant correlation was present for *IGF-1* or *MYOSTATIN* for myofibrillar protein synthesis rates or muscle mass in older adults[101].

In 2004, Willoughby[118] examined the effects of heavy resistance training on muscle strength, muscle mass, serum cortisol, *MYOSTATIN* and *FLRG*, myofibrillar protein as well as mRNA and protein expression of *MYOSTATIN*, *ACTIVIN IIB* receptor and *GLUCOCORTICOID RECEPTOR* in young untrained males (n = 22, 20.87 ± 2.76

yr). Participants were randomly assigned to a progressive resistance training or control group. The resistance trained group exercised 3 days per week for 12 weeks. Training consisted of three sets of six to eight repetitions at 85-90% of 1 RM on leg press, leg extension and leg curl exercises. Testing was conducted at three periods during the course of the study, at baseline and at 6 and 12 weeks post-exercise. Blood samples were obtained immediately prior to and following each testing session while muscle samples were obtained prior to and within 15 minutes following each testing session. Resistance training resulted in a significant increase in total body mass following 12 weeks of training. While there was a significant increase in thigh volume and thigh mass at 12 weeks compared to baseline and following 6 weeks of training. There was a significant increase in lower body relative strength and myofibrillar protein content at 6 and 12 weeks in the training group. Furthermore, at 6 and 12 weeks relative strength and myofibrillar protein content were significantly greater in the training compared to the control group. Serum myostatin, skeletal muscle *MYOSTATIN* mRNA expression and skeletal muscle myostatin protein were each significantly increased at 6 and 12 weeks post-exercise in the training group and were significantly greater in the training group compared to the control group at 6 and 12 weeks post-exercise. There was no apparent trend for the mRNA expression of the *ACTIVIN IIB* receptor or pre-exercise cortisol content over the course of the investigation. However, post-exercise serum cortisol content was significantly increased at 6 and 12 weeks post-exercise and were significantly higher in the training group compared to the control at 6 and 12 weeks. Finally, protein content for glucocorticoid receptor was significantly increased at 6 and 12 weeks in the training group with the increase at 12 weeks being significantly greater

than baseline and 6 weeks. At 12 weeks there was significantly more glucorticoid receptor protein in the training compared to the control group.

In 2007, Hulmi et al.[113] examined the acute and chronic effects of resistance exercise on a select number of myostatin pathway and satellite cell cycle genes in 11 older adults (60.9 ± 5.0 yr). A baseline muscle biopsy was obtained prior to exercise, then participants performed an exercise bout consisting of 5 sets of 10 repetitions on the leg press with 2 minute recovery periods between sets and muscle biopsies were obtained 1 hr and 48 hr post-exercise. Participants then engaged in a whole body, progressive, resistance training program which consisted of two training sessions per week for 21 weeks. To assess chronic adaptations in gene expression muscle biopsies were obtained 30 minutes prior to, 1 hr and 48 hr following the final training session. The mRNA expression of *MYOSTATIN*, *ACTIVIN IIB*, *FLRG*, *P27*, *MYOD* and *MYOGENIN* were examined following an acute bout of resistance exercise prior to and following 21 weeks of resistance training. There was no significant effect on *MYOSTATIN* following an acute bout of exercise, but *MYOSTATIN* mRNA expression was significantly greater prior to exercise following 21 weeks of training compared to baseline. Following chronic resistance training *MYOSTATIN* mRNA expression was significantly downregulated at 48 hr post-exercise compared to pre-exercise *MYOSTATIN* mRNA expression. Following an acute bout of resistance exercise *ACTIVIN IIB* receptor mRNA was significantly downregulated 1 hr post-exercise. There was no significant effect of chronic resistance training on *ACTIVIN IIB* receptor gene expression. No significant effect was found for *FLRG* or *P27* following an acute bout of training or following chronic training. *MYOD* tended to increase 48 hr ($p = 0.09$) following acute resistance training and there was a

trend increase in *MYOD* following chronic testing at baseline, 1 hr and 48 hr post-exercise. MYOGENIN was significantly upregulated 48 hours following acute exercise. There was also a significant upregulation in baseline *MYOGENIN* expression following 21 weeks of training and a significant upregulation in *MYOGENIN* 48 hr post-exercise following chronic resistance training.

Hulmi et al.[114] examined the acute and chronic effects of resistance exercise with or without protein consumption on skeletal muscle hypertrophy and gene expression in young men. Participants were randomly assigned to a protein (n = 11; 25.2 ± 5.2 yr), placebo (n = 10; 27.2 ± 3.0 yr) or control group (n = 10; 24.9 ± 2.7 yr). Participants in the protein group consumed 15 g of whey protein isolate while participants in the placebo group consumed an equivalent amount of a non-energetic placebo prior to and immediately following each resistance training session. The control group did not consume a supplement and did not exercise for the duration of the investigation. Skeletal muscle biopsies were obtained at baseline, 1 hr and 48 hr following an exercise bout consisting of 5 sets of 10 repetitions on a leg press. At 1 hr post-exercise *MYOSTATIN* mRNA expression was found to significantly decrease in the placebo group and non-significantly increase in the protein group. Following a similar trend as *MYOSTATIN*, 1 hr post-exercise *ACTIVIN IIB* receptor mRNA was found to non-significantly decrease in the placebo group and non-significantly increase in the protein group. However, at 48 hr post-exercise *ACTIVIN IIB* mRNA expression was significantly decreased in the placebo and protein groups. Furthermore, in the protein group *CDK2* and *P21* mRNA expression were found to be significantly upregulated 1 hr post-exercise, while *P21* mRNA expression was found to be significantly upregulated in the placebo group at 1 and 48 hr

post-exercise. Finally, *MYOGENIN* was found to significantly decrease 1 hr post-exercise in the placebo group.

To examine the chronic effects of resistance exercise with or without protein consumption on skeletal muscle hypertrophy and gene expression following chronic resistance training participants began a whole body progressive resistance training program in which training was required 2 day per week for 21 weeks. The resistance training program was effective at stimulating skeletal muscle growth and hypertrophy as the protein and placebo groups each experienced a significant increase in body mass, cross-sectional area of the quadriceps femoris and vastus lateralis (protein group experienced a significantly greater increase than the placebo group for the vastus lateralis) determined by MRI analysis and strength determined by 1 RM leg press and isometric knee extension, knee flexion and bench press performance. For the analysis of gene expression of proteins controlling skeletal muscle hypertrophy a final biopsy was obtained 4 to 5 days following the final resistance training session and the results were compared to the baseline muscle sample. Following chronic resistance training there was a non-significant decrease in *MYOSTATIN* mRNA expression in the protein and placebo groups. There was a non-significant increase in the protein group and a non-significant decrease in the placebo group for the mRNA expression of *ACTIVIN IIB* receptor, *MAFBX* and *CDK2*. While there was a non-significant increase in the mRNA expression of *P21* in the protein and placebo groups following chronic resistance training[114].

The results regarding the effect of chronic exercise on myostatin expression have been equivocal. For instance, Walker et al.[117] found a significant decrease in plasma myostatin following 10 weeks of training while Willoughby[118] found a significant

increase in plasma myostatin at 6 and 12 weeks following of resistance training. In regard to intramuscular myostatin expression Roth et al.[23] found a significant decrease in *MYOSTATIN* mRNA expression following 9 weeks of training and Hulmi et al.[114] found a non-significant decrease in *MYOSTATIN* mRNA expression following 21 weeks of chronic resistance training. In contrast Hulmi et al.[113] found *MYOSTATIN* mRNA expression to be significantly upregulated following 21 weeks of chronic resistance training, using the same protocol as the previous investigation[114]. Additionally, Willoughby[118] found a significant increase in *MYOSTATIN* mRNA and protein expression following 6 and 12 weeks of resistance training. Differences in age and/or gender do not account for the equivocal findings regarding resting measures of myostatin following chronic resistance training. Furthermore, differing exercise protocols are likely not the explanation as Hulmi et al.[113] and Hulmi et al.[114] used an identical training protocol and obtained equivocal results regarding *MYOSTATIN* mRNA expression (Table 2).

The equivocal results regarding the effects of chronic exercise on resting myostatin expression may be accounted for by differences in baseline levels of myostatin between participants in the various investigations. Roth et al. found participants with lower baseline *MYOSTATIN* mRNA expression were more likely to experience little to no change in *MYOSTATIN* mRNA following chronic resistance training[23]. Given the small sample sizes of the investigations examined, varying baseline *MYOSTATIN* mRNA expression may account for the equivocal findings. Timing is another issue that may explain the equivocal results between the investigations as muscle samples were obtained at varying time-points following chronic resistance training. For instance, Roth et al.[23]

collected the post-training biopsy 48-72 hr following the final bout of chronic resistance training which is problematic as *MYOSTATIN* mRNA expression has since been found to be significantly downregulated 48 hr following resistance exercise[24, 119] while no found research has examined *MYOSTATIN* mRNA expression 72 hr post-exercise. While other investigations obtained post-training biopsies 3-5 days following the final resistance training bout[113, 114]. Finally, there are six known binding proteins for myostatin: myostatin propeptide, GASP1, FLRG, HSGT, titin cap and follistatin[11] but the only binding protein examined in any of the investigations under review was *FLRG*[24, 118]. As a result the amount of "free" myostatin (myostatin – the myostatin binding proteins) may provide a more reliable measure of the myostatin response following exercise than *MYOSTATIN* mRNA expression alone.

Potential Clinical Applications for Myostatin Inhibition in Diseased Populations

Myostatin inhibition in has been suggested to positively influence skeletal muscle mass[18, 19, 94, 96], maintain a healthy amount of body fat[85, 86, 94] and positively influence glucose sensitivity in animals genetically prone for hyperglycemia[85] or consuming a high fat diet[86]. As a result of the ability of myostatin inhibition to increase skeletal muscle mass scientists have examined the potential relationship between myostatin and HIV[22] along with the effects of myostatin inhibition on disease states characterized by skeletal muscle wasting including muscular dystrophy[18, 120] and amyotrophic lateral sclerosis (ALS)[121].

In 1998, Gonzalez-Cadavid et al.[22] examined myostatin expression in skeletal muscle and serum of healthy men, HIV infected men who lost less than 10% body weight

in the preceding 6 months and HIV infected men who lost at least 10% body weight in the preceding 6 months. Serum concentrations of myostatin immunoreactive protein concentrations (pure mystatin was not yet available so synthetic peptide B was used as a reference standard) were found to be inversely correlated with fat free mass (r = -0.30, p = 0.007) in healthy and HIV infected men. The HIV infected subjects in each group were found to have significantly higher plasma myostatin concentrations compared to the healthy controls. A trend was also present as serum myostatin concentrations were highest in subjects who lost at least 10% body weight in the preceding 6 months. As a result subjects with the highest plasma myostatin concentrations experienced the greatest muscle loss. While western blot analyses suggested that intramuscular myostatin levels were greater in HIV infected subjects compared to the healthy controls.

In 2002, Wagner et al.[18] examined the effects of myostatin deficiency on muscular dystrophy in *mdx* mice, which is a genetic ortholog of Duchenne and Becker muscular dystrophies. Comparisons were made between MSTN$^{-/-}$, *mdx* (n = 12) and MSTN$^{+/+}$, *mdx* mice (n = 22) at 3, 6 and 9 months of age. MSTN$^{-/-}$, *mdx* mice had significantly more body mass, muscle mass and were significantly stronger than MSTN$^{+/+}$, *mdx* mice. More importantly MSTN$^{-/-}$, *mdx* showed less fibrosis and fatty remodeling in diaphragm muscles compared to MSTN$^{+/+}$, *mdx* mice suggesting improved muscle regeneration. However, work from Li et al.[120] suggest that even though myostatin inhibition functions to maintain skeletal muscle mass and increase satellite cell activity in mice with laminin-deficient congenital muscular dystrophy (dy^w/dy^w). The effects of myostatin inhibition also resulted in dy^w/dy^w, MSTN$^{-/-}$ mice having significantly lower amounts of brown and white adipose tissue compared to dy^w/dy^w mice

which may hinder thermoregulation and increase postnatal mortality. Suggesting myostatin inhibition by use of myostatin antagonists following birth may be more effective than knocking out the myostatin gene for the potential treatment of muscular dystrophy.

Myostatin inhibition has also been examined as a potential treatment for ALS[121]. Rats and mice expressing the human SOD1^{G93A} (an ALS disease model) treated with RK35 (an anti-myostatin antibody) were significantly stronger and had more muscle mass than SOD1^{G93A} rats and mice. However, myostatin inhibition did not delay disease onset or extend the survival of rats or mice expressing SOD1^{G93A}. As a result myostatin inhibition was unable to protects against the onset and progression of ALS, but may be able to promote skeletal muscle function in humans with ALS.

Although myostatin inhibition does not appear to be a remedy for muscular dystrophy [120] or ALS[121] the finding that mysotatin null mice have reduced numbers of type I fibers and an increase in the amount of type II fibers compared to normal mice suggests that myostatin inhibition may be useful in the treatment of myopathies resulting from non-genetic causes such as malnutrition, cachexia and corticosteroid excess which are characterized by type II fiber atrophy[120].

CHAPTER III

METHODS

Participants

In 2006, Raue et al.[25] reported a significant difference between younger (23 ± 2 yr) and older women (85 ± 1 yr) for the mRNA expression of *MYOSTATIN* at baseline (younger = 1.90 ± 0.612 AU; older = 2.70 ± 0.566 AU) and 4 hr following an acute bout of resistance exercise. From the data reported by Raue et al.[25] the sample size calculations for a between-subjects study design yielded a minimum sample size of n = 8 for each group in order to attain a statistical power of 0.80. As a result a total of 10 older (60-75 yr) and 10 younger (18-25 yr) males who were not currently participating in any form of resistance training were recruited for this investigation. Futhermore, potential participants met the following inclusion criteria: 1) Participants were apparently healthy and could not have participated in a structured resistance exercise regimen consistently (i.e., at least one time per week) one year prior to participation in this study. 2) Participants abstained from smoking, alcohol, tobacco and caffeine for the duration of the study. 3) Participants did not consume ergogenic, nutritional supplements for at least three months prior to the start of the investigation. 4) Participants were not affected with metabolic disorders including heart disease, arrhythmias, diabetes, thyroid disease or hypogonadism. 5) Participants did not have a history of pulmonary disease, hypertension, hepatorenal disease, clotting disorders, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers or anemia. 6) Participants did not have a previously diagnosed neuromuscular disease that would have prevented them from participating in the resistance training sessions. 7) Participants were not taking prescription medications indicated for heart, pulmonary,

anti-coagulant, anti-hypertensive, psychotropic, neuromuscular/neurological, or androgenic dysfunctions. 8) Participants did not have any absolute or relative contraindication for exercise testing as outlined by the American College of Sports Medicine (listed in the delimitations section). This study was approved by the University of Oklahoma Health Sciences Center Institutional Review Boards for Human Subjects and all participants completed a written informed consent form (Appendix A) and a pre-study health and exercise status questionnaire (Appendix B). All participants in the older group were required to provide written medical clearance from their primary care physician using the form provided (Appendix C).

Research Design

An overview of the research design is presented in Figure 1. A 2 x 4 (group: younger and older males; time-point: T1, T2, T3, T4) repeated measures (RM) design was used to determine the effects of age and repeated bouts of conventional lower body resistance training on myostatin pathway signaling by examining the mRNA expression of *MYOSTATIN*, *ACTIVIN IIB*, *HSGT*, *TITIN CAP*, *FOLLISTATIN*, *FLRG* and *SMURF1*. Additional analyses included the protien expression of pSmad3. All participants visited the laboratory seven times (T1 = day 0 - baseline, FAM1 = day 2, FAM2 = day 4, workout 1 = day 7, T2 = day 9, T3 = day 11, T4 = day 12). Prior to the first visit, participants were be verbally screened to ensure that each person met the inclusion criteria. Furthermore, participants completed the informed consent and health and history questionnaire prior to the first visit. During the first visit (T1), participants reported to the laboratory between 0600 and 0900 following a 12-hour fast and a percutaneous muscle biopsy was obtained from the lateral aspect (i.e., *vastus lateralis*) of their thigh.

Following the biopsy, participants warmed-up for 5 minutes on a cycle ergometer (i.e., 60 rpm at a self-selected intensity) and performed a 1 RM strength test for bilateral leg press, hack squat and leg extension using guidelines established by the National Strength and Conditioning Association[122]. For the leg press and hack squat exercises, a successful repetition required that each participant attained 90º knee flexion at the bottom (eccentric) portion of the repetiton which was visually gauged by a laboratory technician. Two days following T1, participants returned to the laboratory for the first familiarizatio session (FAM1) whereby participants warmed-up for 5 minutes on a cycle ergometer and performed 2 sets of 10 repetitions at a lifting intensity of 60% 1 RM for each of the three lower-body exercises with 2-3 minute rest period between sets. Two days following FAM1, participants returned to the laboratory for the second familiarization session (FAM2) whereby participants warmed-up for 5 minutes on a cycle ergometer and performed 2 sets of 10 repetitions at a lifting intensity of 70% 1 RM for each of the three lower-body exercises with 2-3 minute rest periods between sets. The familiarization sessions were performed for several reasons: 1) To ensure that participants were gradually introduced to the training protocol employed during the training sessions. 2) To minimize the delayed onset of muscle soreness, which may have increased particpant retention. 3) To help ensure each participant was entering the training protocol on a similar baseline exposure to resistance exercise. 4) To help establish proper lifting form with submaxiamal loads prior to intiation of the training protocol.

Two days following FAM2, participants reported to the laboratory for the first training session between 0600 and 0900 following a 12-hour fast. Participants warmed-up for 5 minutes of a cycle ergometer and performed 3 sets of 10 repetitions at a lifting

intensity of 80% 1 RM for each of the three lower-body exercises with 2-3 minute rest periods between sets. Two days following the first training session, participants reported to the laboratory for the second training session (T2) between 0600 and 0900 following a 12 hr fast, donated a second muscle biopsy from the opposite leg of T1, warmed-up for 5 minutes on a cycle ergometer and performed 3 sets of 10 repetitions at a lifting intensity of 80% 1 RM for each of the three lower-body exercises with 2-3 minute rest periods alotted between sets. Two days following T2, participants reported the laboratory for the third training session (T3) between 0600 and 0900 following a 12 hr fast, donated a third muscle biopsy from the opposite leg of T2, warmed-up for 5 minutes on a cycle ergometer, and performed 3 sets of 10 repetitions at a lifting intensity of 80% 1 RM for each of the three lower-body exercises with 2-3 minutes alotted between sets. One day following T3, participants reported to the laboratory for T4 between 0600 and 0900 following a 12 hr fast and donated a fourth muscle biopsy from the opposite leg of T3. During each visit participants were verbally encouraged during each set by a laboratory technician. Furthermore, if a set was not completed during any of the exercise sessions due to volitional fatigue, then 10-20 lbs was removed from the training apparatus and the participant resumed the set until 10 repetitions were completed.

Variables

The independent variable in this investigation was age group (younger vs older adults). The following genes and protein were examined at each muscle biopsy time point and served as the dependent variables.

Muscle mRNA Transcripts

1. *MYOSTATIN*: T1-T4 for each participant. A negative regulator of skeletal muscle mass. Myostatin has been suggested to regulate skeletal muscle mass by inhibiting satellite cell activation. Myostatin also plays a role in the catabolism of skeletal muscle mass through a glucocorticoid mediated response.

2. *ACTIVIN IIB*: T1-T4 for each participant. This receptor has the highest binding affinity for myostatin in skeletal muscle. Myostatin binding results in receptor Smad2/3 phosphoralization and binding with the common Smad4 which translocate the nucleus and influence genes involved with satellite cell regulation.

3. *FOLLISTATIN*: T1-T4 for each participant. Is a myostatin binding protein expressed in skeletal muscle which inhibits myostatin receptor binding.

4. *FLRG* (*FSTL3*): T1-T4 for each participant. Is the primary binding/inhibiting protein of myostatin, which inhibits myostatin receptor binding.

5. *HSGT*: T1-T4 for each participant. Is a myostatin binding protein expressed in skeletal muscle which inhibits myostatin secretion and activation.

6. *TITIN CAP*: T1-T4 for each participant. Is a myostatin binding protein expressed in skeletal muscle which inhibits myostatin latent complex formation and secretion

7. *SMURF1*: T1-T4 for each participant. Is an E3 ubiquitin ligase that tags Smads2/3 for degradation.

Muscle Protein

1. pSmad3: T1-T4 for each participant. The upregulation of myostatin and subsequent binding to the activin IIb receptor results in the phosphorylation of Smad2/3 and subsequently form aggregates with Smad4 to translocate the nucleus and inhibit satellite cell proliferation and differentiation.

Instrumentation

1. *Leg press/hack squat combo (Model #: HLS – 160, Yukon Fitness Equipment, Cleveland, OH).* This machine was used to 1 RM test participants for the leg press and hack squat exercises. This machine was also used to train participants during subsequent workouts for leg press and hack squat exercises.

2. *Leg extension machine (Model #: Nautilus NT 1220 Rotary Leg Extension/Curl Station, Nautilus Inc., Vancouver, WA).* This machine was used to 1 RM test participants for the leg extension exercise. This machine was also used to train participants during subsequent workouts for the leg extension exercise.

3. *Fluorometer (Model #: Versafluor Fluorometer, Bio-Rad Laboratories, Hercules, CA).* This device was used to quantitate DNA from crude muscle homogenates with Hoechst 33258 dye (Sigma, St Louis, MO) used as the probe.

4. *Automated electrophoresis platform (Model #: Experion Electrophoresis Station, BioRad Laboratories, Hercules, CA).* This device was used to quantitate total RNA from the aqueous fraction of the Tri reagent (Sigma, St. Louis, MO) homogenates.

5. *Thermal cycler (Model #: MyiQ Optics Model, Bio-Rad Laboratories, Hercules, CA).* This device was used to quantitate baseline and fold-changes in the mRNA expression of the genes of interest from reverse transcribed mRNA present in the total RNA pool (a.k.a., cDNA) with SYBR green (Bio-Rad Laboratories, Hercules, CA) and gene-specific primers (Integrated DNA Technologies, Coralville, IA) used as probes.

6. *Electrophoresis cell (Model #: Criterion Cell, Bio-Rad Laboratories, Hercules, CA).* This device was used to separate proteins from the cell lysis homogenates based upon

molecular mass. Following this step, the muscle-specific protein (pSmad3) was electrotransferred onto nitrocellulose membranes and probed for transfer efficiency using Ponceau S stain (Sigma, St. Louis, MO). The protein of interest was then immunoprobed using protein-specific antibodies and enhanced using a chemiluminescent reagent (Bio-Rad Laboratories, Hercules, CA).

7. *Electrotransfer cell* (*Model #: Criterion Blotter, Bio-Rad Laboratories, Hercules, CA*). This device was used to transfer proteins from SDS-PAGE gels to nitrocellulose membranes as mentioned above.

8. *Gel documentation system* (*Model #: Chemi Doc XRS, Bio-Rad Laboratories, Hercules, CA*). This device was used to detect and quantitate protein banding to determine muscle-specific protein expression patterns of the protein of interest (pSmad3).

Percutaneous Muscle Biopsies

Over the course of this investigation four biopsies (T1-T4) were obtained from each participant. A baseline (T1) muscle sample was obtained 15 minutes prior to the first familiarizatin exercise bout. The second bioposy was obtained 48 hr following the first training session and 15 minutes prior to the second training session. The third biopsie was obtained 48 hr following T2 and 15 minutes prior to T3. The final biopsie was obtained 24 hr following T3. All biopsies were collected halfway between the hip and patella at a depth between 4 to 5 cm. To minimize repeated biopsy-induced muscle damage[123], biopsies were collected from alternating legs during subsequent testing sessions in such a way that two muscle collections were obtained from each leg. To

minimize the effect of muscle fiber distrabution the second bioposie on each leg was

collected immediately distal to the initial biopsy insertion. The multiple muscle collection

procedures were standardized by using anatomical palpation, visual identification of the

previous biopsy, and depth markings on the needle. Prior to each biopsy, the area was

shaven to remove all hair, and a small area of the skin approximately 2 cm in diameter

was anesthesized with a 1.5 ml subcutaneous injection of 1% Lidocaine HCl. Following

15 minutes, the biopsy area was sterilized in a circular fashion to a 3-inch radius with an

antiseptic soap (i.e., betadine), an incision approximately one-quarter of an inch was

made using a sterile razor and a sterilized 5-mm Bergstrom biopsy needle with suction

applied to its end was inserted into the pilot hole. Suction was applied and muscle tissue

was excised in a double-chop fasion. Immediately following the biopsy, the muscle tissue

was removed from the Bergstrom needle using sterile instruments and the collected tissue

was placed into a labeled cryogenic storage tube and dropped in storage container filled

with liquid nitrogen (i.e., flash frozen). Samples were then transferred into a -80 C freezer

for long-term storage until completion of the investigation at which time samples were

thawed for biochemical analyses.

Muscle [total RNA] determination

A section of muscle from each participant at each time point was weighed (~30

mg) and homogenized using 500 µl of Tri reagent (Sigma Chemical Co., St. Louis, MO)

and a tight-fitting pestle. Following thorough homogenization, samples were centrifuged

at 12,000 revolutions per minute at 4 C for 10 minutes and the resulting supernatant (free

of insoluble protein and high molecular weight DNA) was poured into a new

microcentrifuge tube. Approximately 100 µl of chloroform was added to these samples

and was vortexed for 15 seconds. Then samples were incubated at room temperature for 10 minutes. Samples were then centrifuged at 12,000 revolutions per minute at 4 C for 15 minutes. The upper aqueous phase (containing total RNA) was transferred into a new microcentrifuge tube and 250 µl of 100% isopropanol was used to precipitate the RNA from the aqueous phase. Samples were then centrifuged at 12,000 revolutions per minute at 4 C for 15 minutes to form a RNA pellet and the resulting supernatant was disposed. Then 750 µl of 75% ethanol wash was added to the RNA pellet and samples were be centrifuged at 12,000 revolutions per minute at 4 C for 5 minutes and the resulting supernatant was be disposed. Then the RNA pellet was dissolved in 50 µl of RNase-free water and vortexed. The diluted RNA samples was then stored at -80 C until later analyses.

Total RNA concentrations for each sample were determined using a high sensitivity RNA analysis kit with the Experion Automated Electrophoresis platform (Bio-Rad Laboratories, Hercules, CA). This method separates and quantitates mRNA ranging from 50-6,000 nucleotides in length using a laser-excitable RNA stain and RNA ladder provided by the manufacturer. Furthermore, this procedure has been previously shown in our laboratory to yield un-degraded RNA, free of DNA and proteins as indicated by prominent *28S* and *18S* ribosomal RNA bands (Figure 2). The preparation of reagents and the RNA ladder were performed according to the manufacturer's instructions. Furthermore, all RNA samples and the RNA ladder were thawed on ice during the assay to preserve mRNA integrity and all assays were performed in duplicate.

Real-Time PCR to detect post-exercise expression of genes of interest

Following total RNA concentration determination, 50-200 ng of total RNA was reverse transcribed to synthesize cDNA. For each sample, a reverse transcription reaction mixture (40 µl total) was prepared containing: 1) 200 ng of total cellular RNA diluted to 30 µl with RNase-free water, 2) 8 µl 5x reverse transcription buffer, a dNTP mixture containing dATP, dCTP, dGTP, and dTTP, MgCl$_2$, RNase inhibitor, an oligo(dT)$_{15}$ primer, and 3) 2 µl of MMLV reverse transcriptase enzyme (Bio-Rad Laboratories, Hercules, CA)] were incubated at 42 C for 40 minutes, heated to 85 C for 5 minutes, and then quick-chilled on ice yielding the cDNA product. Finally, 80 µl of RNase-free water was added to bring the cDNA solutions up to 120 µl, and cDNA solutions were subsequently frozen at -80 C until semi-quantitative real-time PCR was performed. Forward and reverse oligonucleotide primer pairs were constructed using commercially available Beacon Designer software (Bio-Rad Laboratories, Hercules, CA) and synthesized (Integrated DNA Technologies, Coralville, IA) (Table 3). *B2M* and *28S* were used as an internal reference for detecting the relative change in the quantity of target mRNA as *B2M* and *28S* are constitutively expressed prior to and following resistance exercise[124]. Two µl of cDNA were added to each of the separate PCR reactions for *MYOSTATIN*, *ACTIVIN IIB*, *HSGT*, *TITIN CAP*, *FOLLISTATIN*, *SMURF1*, *B2M* and *28S*. Each PCR reaction contained the following mixtures: 12.5 µl of SYBR Green Supermix (Bio-Rad, Hercules, CA) (100 mM KCl mixture, 40 mM Tris-HCl, 0.4 mM of each deoxynucleoside triphosphate, 50 U/µl of iTaq DNA polymerase, 6.0 mM MgCl$_2$, SYBR Green I, 20 nM fluorescein), 1.5 µl of sense and antisense gene-specific primers, and 7.5 µl of nuclease-free dH$_2$O. The PCR reactions were amplified with a thermal

cycler (Bio-Rad Laboratories) whereby the amplification sequence involved an initial 10-minute cycle at 95 C to activate the *Taq* polymerase followed by a 40-cycle period with a denaturation step at 95 C for 15 seconds and primer annealing/extension step at 55 C for 45 seconds. It should be noted that all assays were performed in duplicate, and gene expression data was expressed using $2^{-\Delta CT}$ method where ΔCT = (gene of interest − avg *B2M* and *28S*). The coefficient of variation for values between duplicates were less than 5% for all genes of interest and housekeeping genes.

Immunoblotting procedures

A portion of muscle from each time point for each participant was weighed (~25 mg) and homogenized on ice with 300 μl of cell lysis homogenizing buffer [150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM Tris HCl, pH 7.5 spiked with protease/phosphatase inhibitor cocktails (Sigma, St. Louis, MO)]. Prior to SDS-PAGE, protein concentrations of cell lysis homogenates were determined spectrophotometrically using Bradford reagent (Bio-Rad Laboratories, Hercules, CA) with BSA used as a standard curve. Following protein concentration determinations for each sample, a total of (30-50) μg of protein was diluted in Laemmli sample loading buffer spiked with 5% 2-mercaptoethanol, heated at 95 C for 5 minutes, and loaded on handmade 12% polyacrylamide gels (Bio-Rad, Hercules, CA) for SDS-PAGE. Each run lasted ~1 hr at 150 V until the bromophenol blue tracer dye from the Laemmli buffer ran ~1 cm to the end of the gel. Following SDS-PAGE runs, proteins were eluted from the gels to nitrocellulose membranes using a "wet transfer" in an electrotranfer blotting apparatus (Bio-Rad Laboratories, Hercules, CA) with Towbin electrotransfer buffer. Electrotranfers lasted 1 hr in duration at 100 V and 380-500 mA. Following

electrotransfers, loading efficiencies were determined by staining nitrocellulose membranes with Ponceau S stain (Sigma, St. Louis, MO). Membranes were destained in Tris-buffered saline spiked with 1% Tween-20 followed by immunoprobing experiments.

Immunoblotting was then performed using mouse monoclonal antibodies against muscle-specific pSmad3. Nonspecific binding sites on the nitrocellulose membranes were blocked using TBST spiked with 5% nonfat blocking agent (NFBA) for 50 minutes at room temperature. Membranes were then incubated with one of the previously mentioned primary antibody solution [fold-diluted antibody in 5%NFBA in Tris-buffered saline spiked with 1% Tween-20 (TBST)] for 50 minutes at room temperature. The primary antibody solution was then decanted, membranes were washed two times (10 minutes per wash) with TBST, and membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody solution (10,000-fold dilution in TBST; Bio-Rad Laboratories, Hercules CA) for 45 minutes. Finally, the secondary antibody solution was decanted, membranes were washed two times (10 minutes per wash) with TBST, and membranes were incubated with an enhanced chemiluminescent reagent (Bio-Rad Laboratories, Hercules CA) for 5 minutes. Following this step, membranes were placed in the gel documentation system (Bio-Rad Laboratories, Hercules CA) and immunoprobed band densities were determined using band densitometry.

Anthropometric Data

Participants changed into minimal clothing and were barefoot for the measurement of body mass and height on a calibrated scale and standiometer (Detecto, Webb City, MO). Body mass and height were measured to the nearest 0.05 kg and 0.5 cm, respectively. For measurement of height participants were instructed to stand erect,

inhale deeply, point toes up and look straight ahead. Body composition was assessed

utilizing a three site skinfold equation. All skinfold thickness measurements were

obtained from the right side of the body with a calibrated Lange caliper. Measurements

were obtained according to the recommendations of Jackson and Pollock[125] from the

chest, abdomen and thigh. Body density values were then calculated and utilized to

obtain a measure of percent body fat using the generalized skinfold equation of Jackson

and Pollock[125].

<u>Statistical Analyses</u>

The Shapiro-Wilk statistic was performed for each dependent variable at each

time point to assess the data for normality in the distribution of data points. Independent

samples t-tests were used to examine possible between group differences for normally

distributed data (age, height, weight and percent body fat). Separate two-way (Age:

younger vs. older) x testing session (T1, T2, T3, T4 biopsy) RM ANOVAs were used to

determine main and interactive effects for caloric and macronutrient consumption. The

mRNA and protein data were assessed using non-parametric statistics. Nevertheless,

mRNA and protein data were examined for normality of distribution using the Shapiro-

Wilk statistic ($p < 0.05$). The Mann-Whitney U statistic was used to determine which

condition(s) were significantly different at each time point (synonymous to an

independent samples t-test). The Friedman test was used to detect changes in non-

normally distributed data amongst all conditions over time (i.e., detect a main effect for

time amongst all groups). If the Friedman statistic p-value was < 0.05, then Wilcoxin

signed rank test was performed as a *post hoc* measure to determine which time points

were significantly different within each age group (via the split file function). An alpha of

$p < 0.05$ was used to determine significance for all statistical tests, and all analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL). All data are presented as means ± standard error.

CHAPTER IV

RESULTS

Participant Demographics, Lifting and Dietary Analyses

Healthy younger (n = 10; age: 21.0 ± 0.5 years, body mass: 82.3 ± 4.2 kg, height: 178.4 ± 2.2 cm, body fat percentage: $15.4 \pm 2.9\%$) and older men (n = 10; age: 66.4 ± 1.6 years, body mass: 94.2 ± 3.7 kg, height: 180.9 ± 2.2 cm, body fat percentage: $27.4 \pm 1.8\%$) chose to participate in this investigation. Independent samples t-tests were used to determine between age strength comparisons. Younger men had a significantly greater 1 RM for hack squat (younger: 170.0 ± 10.9 kg, older: 105.8 ± 10.4 kg; $p = 0.001$), leg press (younger: 271.1 ± 14.6 kg, older: 182.7 ± 14.9 kg; $p < 0.001$) and leg extension (younger: 64.8 ± 4.0 kg, older: 46.7 ± 2.8 kg; $p = 0.002$) compared to older men. As a result the cumulative training volume was significantly greater during the training bouts in younger compared to older men (younger: $36,392 \pm 1,894$ kg, older: $23,724 \pm 1,639$ kg; $p < 0.001$). Results from the 3 day food diaries indicated that relative caloric (young $= 33.4 \pm 5.1$ kcal/kg/d, old $= 19.5 \pm 1.9$ kcal/kg/d; $p = 0.038$), protein (young $= 1.6 \pm 0.2$ g/kg/d, old $= 0.8 \pm 0.1$ g/kg/d; $p = 0.030$), carbohydrate (young $= 4.1 \pm 0.6$ g/kg/d, old $= 2.3 \pm 0.3$ g/kg/d; $p = 0.017$) and fat (young $= 1.2 \pm 0.2$ g/kg/d, old $= 0.6 \pm 0.1$ g/kg/d; $p = 0.006$) consumption were significantly greater in younger compared to older men.

Normality Distribution of Dependent Variables

The Shapiro-Wilk statistic was performed on each dependent variable at each time-point to check for normality in distribution (Table 4). Results from the Shapiro-Wilk statistic revealed a non-normal distribution for *FLRG*, *FOLLISTATIN*, SMURF1 and pSmad3.

Pre and Post-Exercise Expression of the Genes of Interest

MYOSTATIN mRNA expression values (means ± SE) relative to the average of *B2M* and *28S* are presented in Figure 3. *MYOSTATIN* mRNA expression values for younger men were: T1 = 0.00028 ± 0.00004, T2 = 0.00024 ± 0.00002, T3 = 0.00020 ± 0.00004, T4 = 0.00022 ± 0.00003. *MYOSTATIN* mRNA expression values for older men were T1 = 0.00028 ± 0.00005, T2 = 0.00021 ± 0.00002, T3 = 0.00020 ± 0.00003, T4 = 0.00016 ± 0.00003. Between group comparisons at each time-point were made using the Mann Whitney U test. Analyses revealed no between group differences existed for *MYOSTATIN* mRNA at each time-point (T1: p = 0.880, T2: p = 0.384, T3: p = 0.935, T4: p = 0.257). A Friedman test revealed there was a main effect for time regarding *MYOSTATIN* mRNA expression values (p = 0.001). As a result Wilcoxin signed rank tests were conducted to examine within-group changes over time in younger and older adults. No significant within group changes occurred in *MYOSTATIN* mRNA expression values in the younger men (p > 0.05) but there was a significant downregulation in *MYOSTATIN* mRNA expression at T3 for older men (p = 0.047) and T4 (p = 0.013) compared to baseline.

ACTIVIN IIB mRNA expression values (means ± SE) relative to the average of *B2M* and *28S* are presented in Figure 4. *ACTIVIN IIB* mRNA expression values for younger men were: T1 = 0.00013 ± 0.00002, T2 = 0.00014 ± 0.00001, T3 = 0.00016 ± 0.00004, T4 = 0.00010 ± 0.00001. *ACTIVIN IIB* mRNA expression values for older men were T1 = 0.00026 ± 0.00008, T2 = 0.00017 ± 0.00003, T3 = 0.00011 ± 0.00001, T4 = 0.00014 ± 0.00002. Between group comparisons at each time-point were made using the Mann Whitney U test. Analyses revealed no between group differences existed for

ACTIVIN IIB mRNA at each time-point (T1: p = 0.082, T2: p = 0.427, T3: p = 0.288, T4: p = 0.199). A Friedman test revealed no main effect for time was present (p = 0.320).

FOLLISTATIN mRNA expression values (means ± SE) relative to the average of *B2M* and *28S* are presented in Figure 5. *FOLLISTATIN* mRNA expression values for younger men were: T1 = 0.00007 ± 0.00003, T2 = 0.00003 ± 0.00001, T3 = 0.00023 ± 0.00012, T4 = 0.00003 ± 0.00001. *FOLLISTATIN* mRNA expression values for older men were T1 = 0.00018 ± 0.00005, T2 = 0.00010 ± 0.00002, T3 = 0.00012 ± 0.00003, T4 = 0.00010 ± 0.00004. The Mann Whitney U test was utilized to identify between group differences at each time-point and analyses revealed there was a significant difference between younger and older men at T1 (p = 0.023) and T2 (p = 0.008), but no significant difference between groups was present at T3 (p = 0.568) and T4 (0.174). A Friedman test revealed no main effect for time was present (p = 0.443).

FLRG mRNA expression values (means ± SE) relative to the average of *B2M* and *28S* are presented in Figure 6. *FLRG* mRNA expression values for younger men were: T1 = 0.00005 ± 0.00001, T2 = 0.00006 ± 0.00001, T3 = 0.00009 ± 0.00003, T4 = 0.00005 ± 0.000004. *FLRG* mRNA expression values for older men were T1 = 0.00013 ± 0.00004, T2 = 0.00008 ± 0.00002, T3 = 0.00006 ± 0.00001, T4 = 0.00011 ± 0.00002. The Mann Whitney U test was utilized to identify between group differences at each time-point and analyses revealed there was a significant difference between younger and older men at T1 (p = 0.038) and T4 (p = 0.005), but no significant difference was present at T2 (p = 0.473) and T3 (p = 0.940). A Friedman test revealed no main effect for time was present (p = 0.423).

HSGT mRNA expression values (means ± SE) relative to the average of *B2M* and *28S* are presented in Figure 7. *HSGT* mRNA expression values for younger men were: T1 = 0.00064 ± 0.00009, T2 = 0.00062 ± 0.00005, T3 = 0.00054 ± 0.00013, T4 = 0.00051 ± 0.00008. *HSGT* mRNA expression values for older men were T1 = 0.00076 ± 0.00009, T2 = 0.00078 ± 0.00015, T3 = 0.00060 ± 0.00011, T4 = 0.00054 ± 0.00012. The Mann Whitney U test was utilized to identify between group differences at each time-point and analyses revealed no between group differences existed for *HSGT* mRNA at each time-point (T1: p = 0.226, T2: p = 0.880, T3: p = 0.762, T4: p = 0.762). A Friedman test revealed there was a main effect for time regarding *HSGT* mRNA expression values (p = 0.010). As a result Wilcoxin signed rank tests were conducted to examine within-group changes over time in younger and older adults; however, no significant within group changes occurred in *HSGT* mRNA expression values in the younger or older group (p > 0.05) compared to the respective baseline values.

SMURF1 mRNA expression values (means ± SE) relative to the average of *B2M* and *28S* are presented in Figure 8. *SMURF1* mRNA expression values for younger men were: T1 = 0.00012 ± 0.00002, T2 = 0.00015 ± 0.00002, T3 = 0.00016 ± 0.00003, T4 = 0.00013 ± 0.00002. *SMURF1* mRNA expression values for older men were T1 = 0.00020 ± 0.00003, T2 = 0.00015 ± 0.00003, T3 = 0.00014 ± 0.00003, T4 = 0.00014 ± 0.00002. The Mann Whitney U test was utilized to identify between group differences at each time-point. Analyses revealed no between group differences existed for *SMURF1* mRNA at each time-point (T1: p = 0.076, T2: p = 0.821, T3: p = 0.496, T4: p = 0.677). A Friedman test revealed no main effect for time was present in regard to *SMURF1* mRNA expression values (p = 0.378).

No data is available for *TITIN CAP* as the gene was too lowly expressed to obtain viable data.

Pre and Post-Exercise Expression of the Protein of Interest

Muscle pSmad3 values expressed in arbitrary density units (ADUs) using means ± SE and are presented in Figure 9. Muscle pSmad values for younger men were: T1 = 0.023 ± 0.006, T2 = 0.024 ± 0.005, T3 = 0.029 ± 0.005, T4 = 0.029 ± 0.006. Muscle pSmad values for older men were T1 = 0.020 ± 0.003, T2 = 0.017 ± 0.006, T3 = 0.015 ± 0.004, T4 = 0.014 ± 0.004. The Mann Whitney U test was utilized to identify between group differences at each time-point and analyses revealed there was a significant difference between younger and older men at T3 (p = 0.012) and T4 (p = 0.010). A Friedman test revealed no main effect for time was present (p = 0.546).

CHAPTER V

DISCUSSION

The purpose of the current investigation was two-fold: to examine if baseline differences in myostatin signaling are present between younger and older men and to examine the effects of short-term chronic resistance training on myostatin signaling in younger and older men. Myostatin is synthesized as a 376 amino acid precursor protein composed of a signal sequence, a N-terminal propeptide domain and a C-terminal domain which is considered the active molecule[81]. Myostatin is secreted in a latent (inactive) form as it is bound to a propeptide (latency-associated peptide). A mature (active) form of myostatin is produced after proteolytic processing between the propeptide domain and C-terminal domain, which produces a N-terminal propetide and the mature form of myostatin (the C-terminal dimer)[11].

Gene expression of proteins that were examined in the current investigation that influence myostatin secretion and binding include: HSGT which inhibits myostatin secretion and binding[1], titin cap which inhibits myostatin latent complex formation and secretion[2], FLRG[3] and follistatin[6] which inhibit myostatin binding to the activin IIB receptor[11]. Mature myostatin binds to the activin IIB receptor which associates with its corresponding type I receptor resulting in an activated heterotetrameric receptor complex, that transphosphorylates the type I receptor. Then the activated receptor complex phosphorylates receptor regulated Smad2/3 proteins that oligomerize with the common Smad (Smad4) which translocate into the nucleus and interact with Smad binding partners in the DNA to regulate gene transcription[126]. While Smurf1 is an E3 ubiquitin ligase which tags Smad2/3 for degradation[11] and also associate with Smad6/7

in a signal dependent manner as Smad6/7 allow Smurfs to mark the activated type I receptor for protesomal degradation[8, 127, 128].

There were no significant between group differences in the mRNA expression of *MYOSTATIN* at baseline or following the three repeated bouts of resistance training ($p < 0.05$). Investigations examining the basal expression of myostatin between younger and older adults have resulted in equivocal findings[23, 25, 26, 101, 119, 129]. Raue et al.[25] found *MYOSTATIN* mRNA to be significantly greater in older (83 ± 1 yr) compared to younger (23 ± 1 yr) women, while other investigations have no found no significant differences in *MYOSTATIN* mRNA expression at baseline between younger and older adults[23, 26, 101, 129]. Results have also been equivocal regarding the effects of resistance training on *MYOSTATIN* mRNA expression but are explained by variations in post-exercise muscle biopsy time-points. Investigations have found a significant downregulation in the mRNA expression of *MYOSTATIN* at 1 hr[24, 107, 114], 2 hr[107], 4 hr[25, 107], 12 hr[107], 24 hr[107, 119, 129] and 48 hr[24, 25, 119] following a bout of resistance training. However, other investigations have found no significant effect of exercise on *MYOSTATIN* mRNA expression at 4 hr[113] and 48 hr[113, 114] following a bout of resistance exercise.

In the current investigation younger men experienced a trend decrease for *MYOSTATIN* mRNA expression 24 hr following T4 ($p = 0.074$) while older men experienced a significant downregulation for *MYOSTATIN* mRNA expression at T3 ($p = 0.047$; 48 hr post-exercise) and T4 ($p = 0.013$; 24 hr post-exercise). *MYOSTATIN* mRNA expression has been found to be significantly downregulated up to 48 hr following a bout of resistance training[24, 25, 119], however, these finding are equivocal[113, 114] and it

is likely that the downregulation of *MYOSTATIN* is more pronounced at 24 hr rather than 48 hr following a bout of resistance training.

There were no significant within ($p > 0.05$) or between group ($p > 0.05$) differences for the mRNA expression of *ACTIVIN IIB*. Previous investigations have found resistance training to have no effect on *ACTIVIN IIB* mRNA expression 1 hr[24] and 48 hr[24, 113] post-exercise in older men, while another investigation found *ACTIVIN IIB* mRNA expression to be significantly downregulated 1 hr[113] following a single resistance training bout and tended to decrease ($p = 0.07$) 1 hr following a resistance training bout proceeded by a 21 week resistance training program[113] in older men. In younger men *ACTIVIN IIB* receptor mRNA values have been found to be unaffected 1 hr following resistance training but were significantly downregulated 48 hr post-exercise[114]. Nevertheless, Willoughby[118] found chronic resistance training for 6 and 12 weeks to have no effect on *ACTIVIN IIB* mRNA values 15 minutes post-exercise in young men. The current investigation appears to be the first to report age has no effect on *ACTIVIN IIB* mRNA expression and demonstrates the genetic response in regard to *ACTIVIN IIB* mRNA expression is similar between younger and older men.

Older men had significantly higher mRNA expression values compared to younger men at baseline for the myostatin binding proteins *FLRG* ($p = 0.038$) and *FOLLISTATIN* ($p = 0.023$). Older men also had higher mRNA expression values for *FLRG* at T4 ($p = 0.003$) and had higher mRNA expression values for *FOLLISTATIN* at T2 ($p = 0.007$). No within group differences were present for younger or older men in regard to *FLRG* or *FOLLISTATIN* ($p > 0.05$). No found literature has examined the effects of age on *FLRG* expression; therefore, the finding in the current investigation that

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older adults have significantly greater mRNA expression values of *FLRG* appear to be novel. However, the effects of resistance training on *FLRG* mRNA expression have been examined[24, 113]. In 2007, Hulmi et al.[113] found the mRNA expression of *FLRG* to be unaffected by resistance training 1 hr and 48 hr post-exercise following an acute bout of resistance training and following 21 weeks of training in older men (63.2 ± 6.3 yr). In 2008, Hulmi et al.[24] found resistance training to have no effect on the mRNA expression of *FLRG* 1 hr and 48 hr following a bout of resistance training. However, participants who consumed 15 g of whey protein isolate immediately before and following a bout of resistance training experienced a significant upregulation of *FLRG* 48 hr post-exercise, suggesting macronutrients may be an effective mechanism to alter myostatin pathway signaling following a bout of resistance training in older men (62.1 ± 4.2 yr)[24].

In regard to *FOLLISTATIN* our results are consistent with the work of Jensky et al.[26] who reported baseline differences in the mRNA expression of *FOLLISTATIN* between younger (28 ± 5 yr) and older (68 ± 6 yr) men. Also consistent with the results from Jensky et al.[26] the current investigation found resistance exercise to have no within or between group effect in the mRNA expression of *FOLLISTATIN* 24 hr following a bout of resistance training. Animal models have been used to demonstrate the importance of follistatin in the myostatin signaling pathway as mice over-expressing follistatin have been found to experience nearly a two-fold increase in skeletal muscle mass compared to wild-type littermates[130]. Moreover, follistatin appears to influence skeletal muscle mass independent of myostatin signaling as myostatin null mice over-expressing follistatin experienced a nearly four-fold increase in skeletal muscle mass

compared to wild-type littermates while myostatin null mice typically experience a near two-fold increase in skeletal muscle mass compared to wild-type controls, suggesting follistatin to influence skeletal muscle mass independent of myostatin pathway signaling[131, 132].

There were no significant within ($p > 0.05$) or between group ($p > 0.05$) differences for the mRNA expression of *HSGT* which is consistent with the results of a previous investigation[26]. We were unable to obtain mRNA expression values for *TITIN CAP*. Previous investigations which have examined the effects of titin cap on myostatin have utilized cell culture techniques [2, 133]. Future investigations should seek to determine the effects of age, exercise and nutrition on titin cap as myostatin and titin cap have been found to have a high protein-protein interaction using surface Plasmon resonance kinetics[2]. Furthermore, when titin cap was overexpressed in C_2C_{12} myoblasts the rate of satellite cell proliferation was significantly increased and contained lower levels of myostatin[2]. In the future researchers looking to examine *TITIN CAP* in human skeletal muscle should use a different primer sequence from the one used in the current investigation as we were unable to obtain a measurable value of the mRNA expression of *TITIN CAP*.

There were no significant within ($p > 0.05$) or between group ($p > 0.05$) differences for the mRNA expression of *SMURF1* which appear to be novel findings as no found literature exists on *in vivo SMURF1* mRNA or protein expression involving myostatin pathway activity. Research has been conducted on the effects of *SMURF1/2* in TGF-β pathway signaling[128, 134, 135], but these investigations have been conducted utilizing cell culture techniques[134, 135]. The over-expression of SMURF1/2 has been

found to decrease TGFβ1-induced GLα promoter activity and to strengthen the inhibitory effect of Smad7 on promoter activity while decreasing Smad3/4 mediated GLα promoter activity suggest that SMURF1/2 can downregulate the TGF-β1 signaling pathway[135]. Since the TGF-β1 signaling pathway function in a similar manner as the myostatin signaling pathway the effects of SMURF1 are likely to function in a similar manner in each pathway. Regardless, the current investigation appears to be the first to demonstrate the lack of effect of age and exercise on the mRNA expression of *SMURF1*.

There were significant between group differences in the protein expression of pSmad3 as older men had significantly less protein for pSmad following T3 (p = 0.012) and T4 (p = 0.010) compared to younger men. This finding appears novel as no research to our knowledge has demonstrated that resistance training influences pSmad3 in human skeletal muscle. However, the finding that pSmad protein expression was significantly decreased at T3 and T4 in older compared to younger men is likely the result of the finding that older men experienced a significant decrease in *MYOSTATIN* mRNA expression at T3 and T4. While the physiological relevance of a decrease in pSmad3 protein expression is difficult to explain in the current investigation this adaptation may occur to facilitate hypertrophic mechanisms in older skeletal muscle as it is known that pSmad3 (activated Smad3) is known to increase the expression of genes that potentially inhibit satellite cell activity (i.e., proliferation and differentiation)[11]. This finding is consistent with previous work which has found genes to promote satellite cell differentiation (*MYF5*, *MYOD*, *MYOGENIN* and *MRF4*) to be more highly expressed in older compared to younger men[25]. Although counterintuitive because skeletal muscle growth occurs more readily in younger compared to older men the results have been

explained by suggesting the pro-hypertrophic genes are expressed to a greater degree in older compared to younger men in an attempt to maintain skeletal muscle mass with age[25].

To date results from the current investigation provide the most complete picture of the effects of age and short-term, chronic resistance training on myostatin pathway signaling. Of particular interest was the finding that at baseline the mRNA expression of the primary components of the myostatin pathway are similar between younger and older men except for the myostatin binding proteins, *FLRG* and *FOLLISTATIN* which were expressed to a greater degree in older compared to younger men. Also of interest was the finding that in terms of myostatin signaling older and younger men responded in a similar fashion in response to the repeated bouts of resistance training. The only difference present between groups was the differential response in *MYOSTATIN* mRNA as the older men experienced a significant decrease 48 hr following T3 ($p = 0.047$) and 24 hr following T4 (0.013) while younger men experience a trend decrease 24 hr following T4 (0.074).

Given the findings in the current and previous investigations which suggest there are no baseline differences in the mRNA expression of *MYOSTATIN* [23, 26, 101, 129] and *ACTIVIN IIB* between younger and older adults combined with the findings that *FLRG* and *FOLLISTATIN* [26] are expressed to a significantly greater degree in older compared to younger adults at baseline suggest that at rest older men may have less biologically active myostatin present in skeletal muscle compared to younger men. However, this hypothesis is limited by several factors: 1) Protein expression of myostatin, FLRG and follistatin were not quantified and previous investigations have demonstrated

there is not a 1:1 ratio from gene transcription to translation[98, 136] and this ratio may be influenced by age. As a result even though gene expression was expressed to a greater degree in older men compared to younger men the possibility exists that no differences in the protein expression of the genes of interest may exist between groups. 2) Protein-protein interactions between FLRG and myostatin and follistatin and myostatin were not conducted. Even if FLRG and follistatin protein were quantified without conducting protein-protein interactions it would not be possible to determine if the myostatin binding proteins (FLRG and follistatin) were binding to myostatin. Finally, future work should examine if differences exist in the binding affinity for myostatin to the activin IIB receptor with age. Collectively, results from the current investigation suggest myostatin pathway signaling to have little influence on the loss of skeletal muscle mass with age as the minor differences found in the mRNA expression of the primary myostatin signaling pathway proteins appear to become more favorable with age as found by the significant upregulation of the myostatin binding proteins, *FLRG* and *FOLLISTATIN*, combined with a more pronounced decrease in the mRNA expression of *MYOSTATIN* following repeated bouts of resistance training in older men.

Even though results from the current investigation suggest myostatin pathway signaling to have little effect on the loss of skeletal muscle mass with age numerous investigations have found that genetically knocking out myostatin[18, 94, 96, 98, 137] and inhibiting myostatin with a myostatin blocking antibody[21, 138] have shown the potential to be a safe[21] and effective mechanism for increasing and maintaining skeletal muscle mass with age[20, 139], in diseased populations[18, 120, 121] and could be useful for the treatment of myopathies resulting from non-gentic causes such as

malnutrition[120], cachexia[120] and corticosteroid excess[15, 16, 99, 100, 120] which are characterized by type II fiber skeletal muscle atrophy[120]. Further investigations have also demonstrated the overexpression of titin cap[2] and follistatin[130] to significantly increase skeletal muscle mass. As a result even though age does not appear to influence myostatin pathway signaling much research exists demonstrating the potential positive effects of altering myostatin pathway activity in the favorable direction can have on the maintenance of skeletal muscle mass with age and those suffering from disease states characterized by skeletal muscle wasting. Therefore, pharmacological interventions designed at shifting the myostatin pathway to promote skeletal muscle growth are currently being and should continue to be examined for the benefit of maintaining quality and enhancing the duration of life of those experiencing skeletal muscle wasting.

From a global perspective understanding the mechanisms involved in myostatin signaling are of importance particularly when considering the practical applications with slowing/reversing the loss of skeletal muscle mass with age. In the United States nearly 50% of older adults ($\geq$ 60 yr) have been estimated to be sarcopenic with approximately 20% being classified as functionally disabled and estimates of the direct health care costs of sarcopenia were estimated to be $18.5 billion in 2000[140]. Currently the most effective mechanisms to slow the rate of skeletal muscle loss with age are resistance exercise[141, 142] and androgen replacement therapy[141]. However, physical activity has been found to decrease with age[143] and testosterone is often widely unavailable and may be associated with adverse effects in older adults[144]. Conversely, research conducted on myostatin null animals[18, 94, 96, 98, 137], mice overexpressing titin

cap[2] and follistatin[130], and mice introduced to a myostatin blocking antibody[21, 138] have clearly demonstrated the profound effect of myostatin signaling on the regulation of skeletal muscle growth and degradation. Furthermore an investigation by Whittermore et al. provides evidence suggesting that short term of administration of a myostatin blocking antibody at a dose of 60 mg/kg/week to be a safe and effective mechanism to enhance skeletal muscle mass without the introduction of adverse effects *in vitro*[21]. Additional work conducted by Siriett et al. found younger and older mice to have an increased rate of skeletal muscle recovery following injury and an increase in the number activated satellite cells compared to wild-type controls[20]. Further evidence of the apparent safety of altering the myostatin pathway is evidenced in the work of Schuelke et al. who conducted a case study of a German boy who was born myostatin null. At 4.5 yr of age the boy had no apparent health problems but was hypoglycemic and had increased serum concentrations of testosterone and IGF-1[145].

Given the findings that alterations in myostatin pathway activity have been found to positively influence fat mass[85-87] and insulin resistance[85, 86] in healthy and diseased populations along with the findings that myostatin inhibition positively influences skeletal muscle mass in diseased[18, 120, 121] and healthy aged populations[20, 21] it is imperative that pharmacological interventions continue to be developed and research continues to explore the safety and efficacy of novel pharmacological agents designed to alter myostatin pathway activity. Of particular interest have been the findings that the introduction of myostatin blocking antibodies[20, 21] in aged mice has been found to enhance muscle regeneration and increase the number activated satellite cells[20] and to be apparently safe[21]. Given the efficacy[20, 21] of

myostatin blocking antibodies along with the apparent safety for short-term use[21] combined with the knowledge that the best clinical treatment to prevent skeletal muscle loss is testosterone administration, which has been found to be associated with adverse effects, it seems logical from a practical and economic standpoint to continue to explore mechanisms to alter myostatin pathway signaling to promote skeletal muscle growth.

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Appendix A
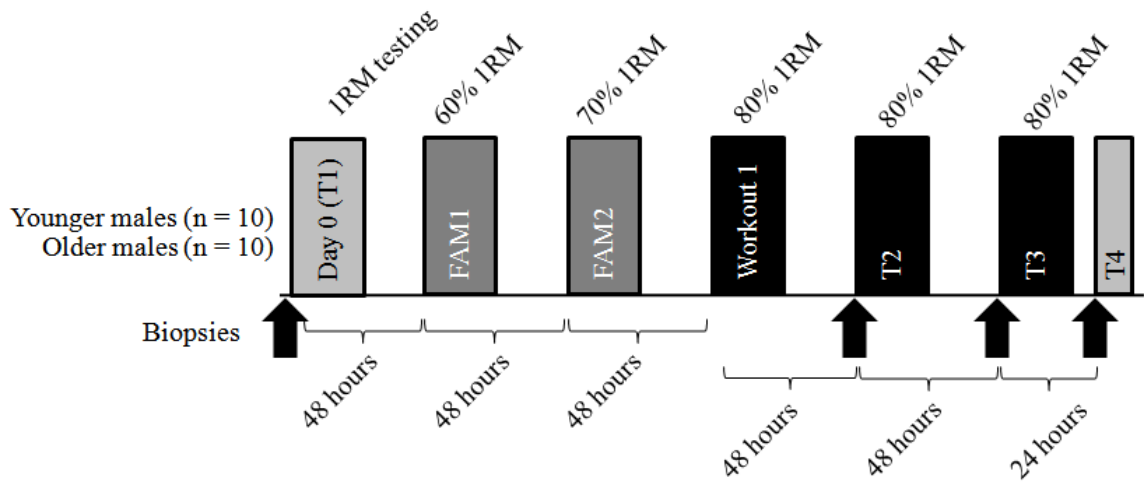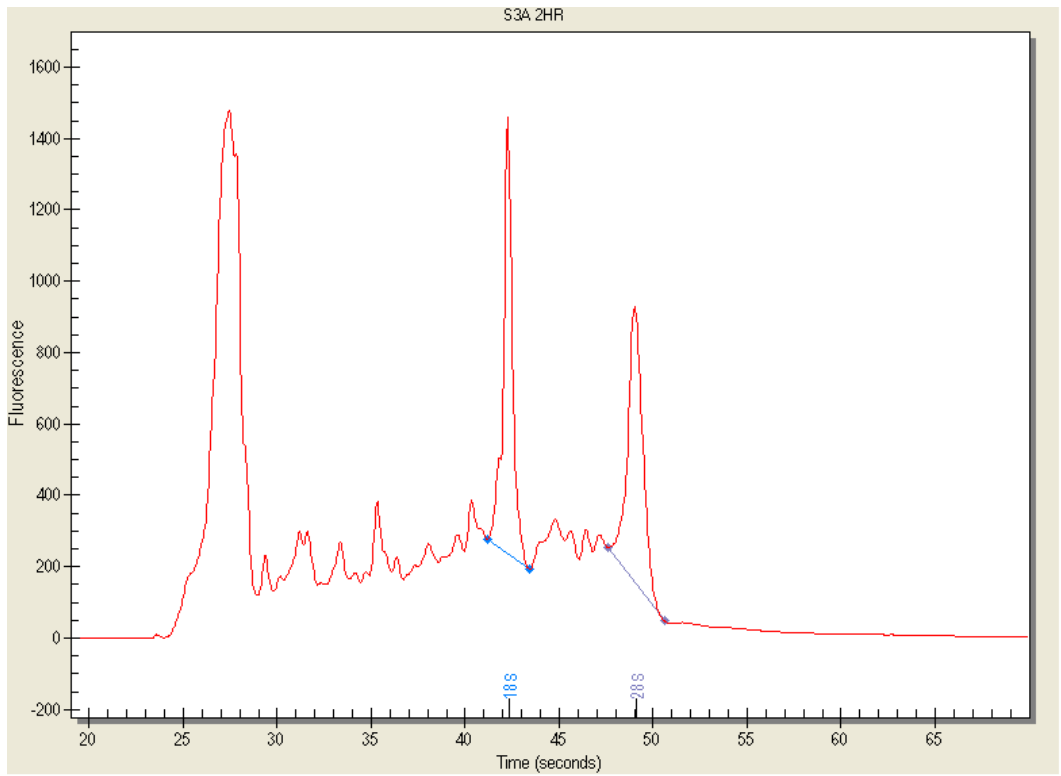
Fragment Number	Fragment Name	Start Time	End Time	Area	% of Total Area
1	18S	41.20	43.45	1631.71	6.42
2	28S	47.65	50.60	1458.26	5.74

RNA Area   25398.80
RNA Concentration   56157.59 pg/µl
Ratio [28S/18S]   0.89

MYOSTATIN mRNA expression relative to mean of 28S & B2M ($2^{-\Delta CT}$)

baseline (T1) 48-h post bout 1 (T2) 48-h post bout 2 (T3) 24-h post bout 3 (T4)

☐ Young (n = 10)   ■ Old (n = 10)

Y-axis: *FLRG* mRNA expression relative to mean of 28S & B2M ($2^{-\Delta CT}$)

X-axis: baseline (T1), 48-h post bout 1 (T2), 48-h post bout 2 (T3), 24-h post bout 3 (T4)

Legend: □ Young (n = 10)   ■ Old (n = 10)

FOLLISTATIN mRNA expression relative to mean of 28S & B2M ($2^{-\Delta CT}$)

baseline (T1) 48-h post bout 1 (T2) 48-h post bout 2 (T3) 24-h post bout 3 (T4)

□ Young (n = 10) ■ Old (n = 10)

Figure axis labels: *SMURF 1* mRNA expression relative to mean of 28S & B2M ($2^{-\Delta CT}$)

Y-axis values: 0.000000, 0.000050, 0.000100, 0.000150, 0.000200, 0.000250

X-axis categories: baseline (T1), 48-h post bout 1 (T2), 48-h post bout 2 (T3), 24-h post bout 3 (T4)

Legend: ☐ Young (n = 10)   ■ Old (n = 10)

Appendix B

Table 1. Time course response of genes influencing satellite cell activity following an acute bout of resistance exercise

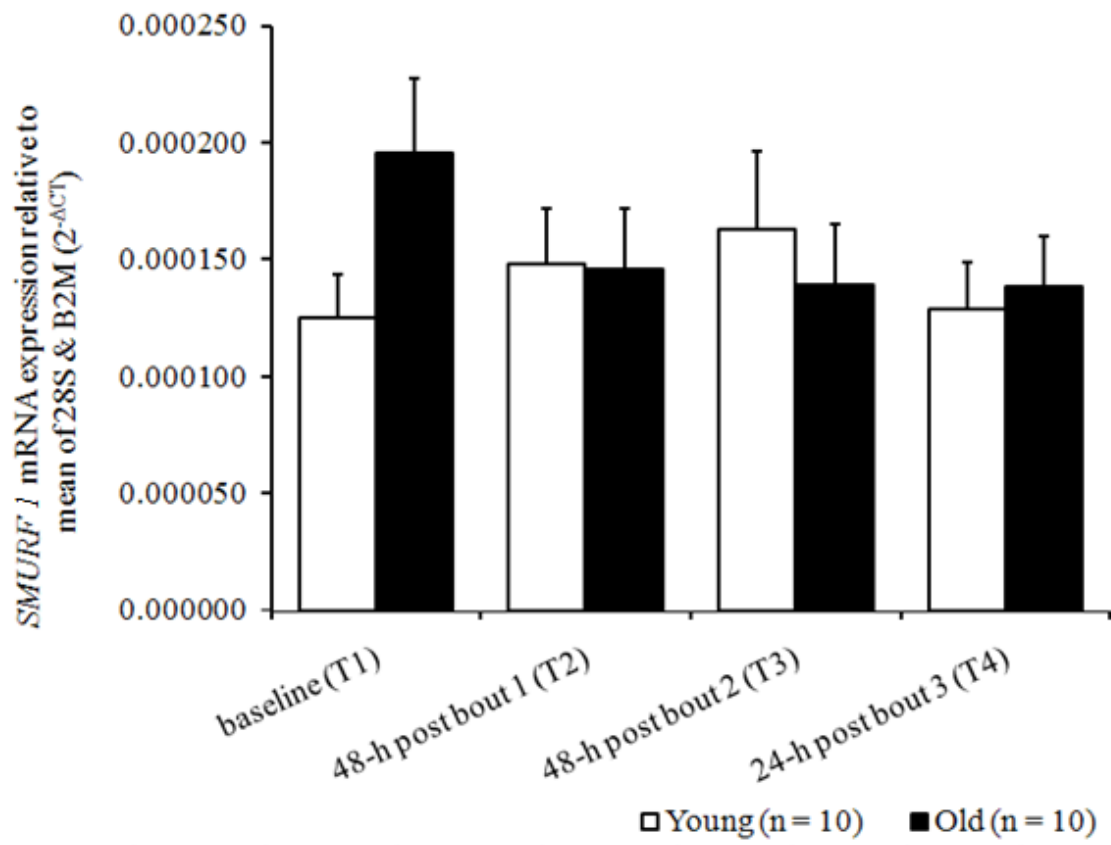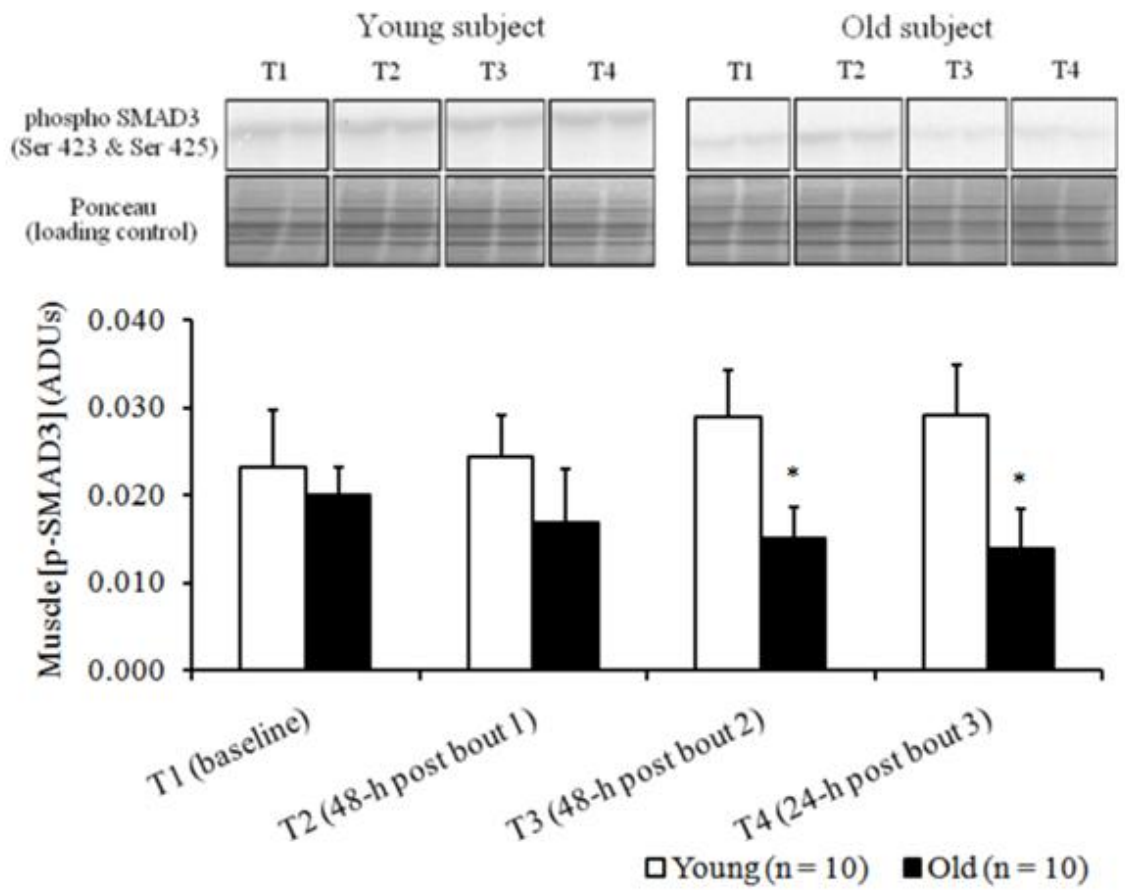| Study	Age	Gender	Gene(s)	Time-point response
Louis et al.[107]	25 ± 4 yr	Men and women	Myostatin	Significant ↓ immediate post, 1, 2, 4, 8, 12, 24 hr post exercise
Raue et al.[25]	Younger: 23 ± 2 yr Older: 85 ± 1 yr	Women	Myostatin	Significant ↓ 4 hr post exercise in young and old
Hulmi et al.[24]	62.1 ± 4.2 yr	Men	Myostatin	Only results from the placebo group are presented Significant ↓ 1 hr and 48 hr post-exercise
Hulmi et al [113]	60.9 ± 5.0 yr	Men	Myostatin	Only results from the placebo group are presented ↔ 4 hr and 48 hr post-exercise
Hulmi et al.[114]	27.2 ± 3.0 yr	Men	Myostatin	Only results from the placebo group are presented Significant ↓ 1 hr post exercise Non-significant ↑ 48 hr post-exercise
Raue et al. [25]	Younger: 23 ± 2 yr Older: 85 ± 1 yr	Women	Muscle Regulatory Factors	The response for each variable was the same in younger and older adults Significant ↑ MyoD and MRF4 4 hr post exercise ↔ Myf5 4 hr post exercise ↔ myogenin 4 hr post-exercise
Hulmi et al. [24]	62.1 ± 4.2 yr	Men	Muscle Regulatory Factors	Only results from the placebo group are presented ↔ myogenin 1 hr and 48 hr post-exercise ↔ myoD 1 hr and 48 hr post-exercise
Hulmi et al [113]	60.9 ± 5.0 yr	Men	Muscle Regulatory Factors	Only results from the placebo group are presented ↔ myoD 4 hr and 48 hr post-exercise
Hulmi et al.[114]	27.2 ± 3.0 yr	Men	Muscle Regulatory Factors	Only results from the placebo group are presented Significant ↓ myogenin 1 hr post-exercise ↔ myogenin 48 hr post exercise
Hulmi et	62.1 ± 4.2	Men	Cell Cycle	Only results from the placebo

al. [24]	yr		Regulators	group are presented ↔ p27 1 hr and 48 hr post exercise ↔ p21 1 hr post exercise Significant ↑ p21 48 hr post-exercise ↔ cdk2 1 hr and 48 hr post-exercise
Hulmi et al [113]	60.9 ± 5.0 yr	Men	Cell Cycle Regulators	Only results from the placebo group are presented ↔ p27 4 hr and 48 hr post-exercise
Hulmi et al.[114]	27.2 ± 3.0 yr	Men	Cell Cycle Regulators	Only results from the placebo group are presented Significant ↑ p21 1 hr and 48 hr post exercise ↔ cdk2 1 hr and 48 hr post exercise

Table 2. Effects of chronic resistance exercise on myostatin expression

Study	Age	Duration of Training	Post Biopsy Time-Point	Effect on Myostatin
Roth et al.[23]	Younger (20-30 yr) Older (65-75 yr)	9 weeks	2-3 days following final training bout	Significant ↓ mRNA in younger and older adults
Walker et al.[117]	Younger (18-45 yr)	10 weeks	After final training session, no specific time-point given	Significant ↓ plasma myostatin
Willoughby[118]	Younger (22.9 ± 2.8 yr)	6 and 12 weeks	Serum and muscle: Immediately before final training bout; should be 24 hr after previous training bout	Significant ↑ plasma myostatin and skeletal muscle myostatin mRNA and protein at 6 and 12 weeks
Hulmi et al.[113]	Older (60.9 ± 5.0 yr)	21 weeks	3-4 days following a strength testing protocol	Significant ↑ mRNA
Hulmi et al.[114]	Young protein (25.2 ± 5.2 yr) Young placebo (27.2 ± 3.0 yr)	21 weeks	4-5 days following final training bout	Non-significant ↓ mRNA in protein and placebo group

Table 3. Primer sequences used to probe genes of interest expressed in skeletal muscle samples

Gene	Primer sequence (forward and reverse)	GenBank accession #
MYOSTATIN	5'- GAC CAG GAG AAG ATG GGC TGA ATC CGT T-3' 5'- CTC ATC ACA GTC AAG ACC AAA ATC CCT T-3'	NM_005259
ACTIVIN IIB	5'- GCC TTG CCA TCA GAT TGT G-3' 5'- GCC ATC AGA ACC AGA TAT ACC -3'	NM_001106
HSGT	5'- TTG GGG TGA CGG TAG AAG AC -3' 5'- GTT GAG CTC GAT GGC TTT TC -3'	NP_AJ223828
TITIN CAP	5'- GAG ACT CCA TCG GTA CTA -3' 5'- CCT TCC TAG ACT GTG ACA -3'	NM_003673
FOLLISTATIN	5'-TGC CAC CTG AGA AAG GCT AC -3' 5'- ACA GAC AGG CTC ATC CGA CT -3'	NM_013409
FLRG	5'- TGC TCA GAA TCG CCT ACC-3' 5'- CTC CGT GTT GTC CTC TCC -3'	NM_005860
SMURF1	5'- TGA AGG AAC GGT GTA TGA AG- 3' 5'- CGG TGC TAT CTG TGT AAG G -3'	NM_020429

HSGT = human small glutamine-rich tetratricopeptide repeat-containing protein
FLRG = follistatin-related gene

Table 4. Normality distribution tests for all dependent variables

Variable	T1 p-value	T2 p-value	T3 p-value	T4 p-value
mRNA				
MYOSTATIN	0.912	0.393	0.968	0.280
ACTIVIN IIB	0.089	0.436	0.315	0.218
HSGT	0.247	0.912	0.796	0.796
*FOLLISTATIN**	0.023	0.007	0.604	0.190
*FLRG**	0.035	0.481	0.971	0.003
*SMURF1**	0.099	0.095	0.008	0.061
Protein				
pSmad3*	0.070	0.004	0.013	0.004

Data represent the Shapiro-Wilk statistic p-values for each dependent variable.
* indicates that data at one or multiple time points from T1-T4 was not normally distributed.

Appendix C

Figure 1. Study Design Overview: All workouts (dark gray and black bars) will consist of a 5-minute warm-up on a cycle ergometer followed by 3 sets of 10 repetitions for the bilateral leg press, hack squat and leg extension exercises (intensities denoted) with 2-3 minutes of rest between sets and exercises.

Figure 2. RNA automated electrophoresis electropherogram. Past data from our laboratory demonstrating that our isolation techniques yields high quality RNA as evidenced by the presence of *18S* and *28S* rRNA peaks and an RNA Quality Indicator (RQI) score of 7.0 as well as the lack of high molecular weight peaks past the *28S* peak (indicative of DNA contamination).

Figure 3. *MYOSTATIN* mRNA expression values expressed as means ± SE. † = Significant within group difference from baseline, $p < 0.05$.

Figure 4. *ACTIVIN* IIB mRNA expression values expressed as means ± SE. There were no between or within group differences, $p > 0.05$.

Figure 5. *FLRG* mRNA expression values expressed as means ± SE. * = Significant between group difference, $p < 0.05$.

Figure 6. *FOLLISTATIN* mRNA expression values expressed as means ± SE. * = Significant between group difference, $p < 0.05$.

Figure 7. *HSGT* mRNA expression values expressed as means ± SE. There were no between or within group differences, $p > 0.05$.

Figure 8. *SMURF1* mRNA expression values expressed as means ± SE. There were no between or within group differences, $p > 0.05$.

Figure 9. pSMAD3 protein expression values expressed as means ± SE. * = Significant between group difference, $p < 0.05$.

Appendix D

Consent Form

University of Oklahoma Health Sciences Center (OUHSC)

University of Oklahoma-Norman

Impact of Age on Insulin and Androgen Receptor Expression and Binding Affinity

After Sequential Sessions of Lower Body Resistance Exercise

Sponsor: Department of Health and Exercise Science, University of Oklahoma

Principal Investigator: **Chad M. Kerksick, PhD**

University of Oklahoma

405-325-9021

This is a research study. Research studies include only patients who choose to take part in them. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?

You are being asked to take part in this study because you are a healthy man who is able to exercise.

Why Is This Study Being Done?

Aging in men is associated with decreases in androgens (male hormones). Testosterone is a type of androgen hormone. Decreased testosterone as a result of aging can lead to changes in muscle, including a loss of muscle and muscle strength. In this study, blood and muscle samples will be collected in order to compare amounts of testosterone and how receptive the muscles are to effects of androgens in younger and older men. The purpose of this study is to assess whether three sessions of lower

extremity strength training changes the receptiveness to androgens in the muscle in elderly men compared to younger men.

What is the Status of the Drugs (Devices or Procedures) involved in this study?

No experimental drugs or foods will be used in this protocol. Lidocaine is approved by the FDA as a local anesthetic.

How Many People Will Take Part In The Study?

About twenty men between the ages of 18-25 and 60-75 years of age will be divided into two groups by their age to take part in this study. All of the participants will complete their testing in the research laboratories in the Huston Huffman Center on the University of Oklahoma-Norman campus.

What Is Involved In The Study?

During this time you will complete one familiarization visit prior to completing five visits to the lab. Each visit is outlined below:

- **Familiarization:** Initial familiarization to the study protocol will occur by phone with one of the study investigators. During this visit, the study investigator will ask you about your family and personal health history in addition to various lifestyle habits, which will include your current alcohol and illegal drug use.
- **Visit #1** –During this visit, your highest level of strength will be determined. Your strength will be determined using three separate weight lifting exercises that will focus on the muscles in your legs. To complete this, low amounts of weight will first be used and the amount of weight will be slowly increased until the highest amount of weight you can lift is determined. You will be allowed to rest for 3 minutes between each attempt at lifting the weight. Trained investigators will be present to instruct you on how to safely and effectively complete the exercises.
- **Visit #2** - Two weeks after visit #1, all participants will return to the laboratory for approximately 60 minutes to complete visit #2. All participants will first complete a warm-up which will consist of walking for 10 minutes at a speed with which you are comfortable walking and lifting weights with amounts that will be half of what you completed at the first testing session. For example, if your maximum amount was 100 pounds, you will warm-up with 50 pounds. After warming up, you will then complete three sets of 8 to 10 repetitions (a repetition is lifting and lowering the weight once in a controlled manner) with all three exercises. The amount of weight you will use will be equal to 80% of your

maximum amount (Example: If your maximum amount was 100 pounds, you will use 80 pounds). You will rest for 3 minutes between each set of exercise. If the weight becomes too great, the amount of weight will be decreased so you can complete all of the repetitions. Prior to beginning this exercise bout, you will have a small sample of muscle tissue removed from the outside portion of your thigh, halfway between your hip bone and your kneecap. The amount of muscle tissue will be equivalent to the size of a lead tip from a No. 2 pencil. During this procedure, your skin will be made numb using the same numbing agent that is used at the dentist. The needle used for this procedure is larger than a needle which is used for drawing blood. A small incision, approximately one-quarter of an inch will be made to more easily insert the muscle collection needle. As mentioned earlier, your skin will be made numb and as a result you will feel very little pain and likely significant pressure while the procedure is being completed. This entire process should take approximately one to two minutes. The muscle collection will be completed by Chad Kerksick, PhD, who is a professor of exercise physiology at the University of Oklahoma. Dr. Kerksick will be assisted by trained graduate students to assist him with this procedure. Prior to beginning the exercise session, immediately after the exercise session and 30 minutes after completing the exercise session, you will have approximately 15 milliliters (one tablespoon) of blood drawn from a vein located in the area in front of your elbow. The needle and supplies used are similar to what is used by your physician's office to draw blood. The blood will be drawn by Chad Kerksick, PhD, or graduate students trained in phlebotomy. It is important for you to follow all instructions provided to you by Dr. Kerksick and his staff to minimize any bruising and/or discomfort you may feel from the muscle collection and blood draw. To ensure your safety and provide medical care, Steven Blevins, MD and Ryan Brown, MD will be available to provide medical consult to Dr. Kerksick and his staff if you experience any unexpected problem. This is important for you to understand since Dr. Brown and Dr. Blevins will not be available on-site for emergencies but will be available for medical consultation for cases of infection, hematomas, etc.

- **Visit #3** – Approximately 48 hours after visit #2, participants will return to the laboratory for approximately 60 minutes to complete their second exercise session, which will be identical to visit #2. Immediately before this exercise session a blood and muscle sample will be collected. Two additional blood samples will then be collected immediately after and 30 minutes after completing this exercise session. All blood and muscle samples collected during this visit will follow the procedures outlined previously in the *Visit #2* section.
- **Visit #4** – Approximately 48 hours after visit #3, participants will return to the laboratory for approximately 60 minutes to complete their third and final exercise session, which will be identical to the two previous exercise sessions. Immediately before this exercise session a blood and muscle sample will be collected. Two additional blood samples will then be collected immediately after and 30 minutes after completing this exercise session. All blood and muscle samples collected during this visit will follow the procedures outlined previously in the *Visit #2* section.

- **Visit #5** – Approximately 24 hours after visit #4, participants will return for their final muscle collection following the previously outlined procedures.

How Long Will I Be In The Study?

This study should last for 21 days. There may be anticipated circumstances under which your participation may be terminated by the investigator without regard to your consent, which include:

- He feels that it is in your medical best interest.
- Your condition worsens.
- New information becomes available.
- The study is stopped by the sponsor.

You can stop participating in this study at any time.

What Are The Risks of The Study?

While on the study, you are at risk for these side effects. You should discuss these with the researcher and/or your regular doctor prior to providing your consent to participate.

Very Likely To Occur

- Feeling faint, lightheaded, or nauseated before, during and immediately after the blood and muscle collection. This is a common response and subsides in most individuals upon completion.
- Pain, bruising, feeling faint and arm soreness from having your blood drawn during the 48 to 72 hours after completion.
- Pain, bruising, feeling faint and muscle soreness from having the muscle collection performed during the 48 to 72 hours after completion.
- Muscle soreness or stiffness from completing maximal strength tests and the exercise sessions during the 48 to 72 hours after completion.
- Shortness of breath during the exercise testing and exercise sessions.

Less Likely To Occur but Serious

- An allergic reaction to Lidocaine.
- Chest pain, heart attack and abnormal heart rhythm during the exercise testing and exercise sessions.

Less Likely To Occur

- Slight risk of infection from having your blood drawn and the muscle collection during the 48 to 72 hours after completion

Are There Benefits to Taking Part in The Study?

There is no direct benefit to you to participate in this study, but the information from this study may increase knowledge about the effects of aging on male hormones, muscle strength, and exercise.

What Other Options Are There?

Your alternative is to not participate.

What About Confidentiality?

Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include faculty members and graduate students appointed to this protocol from the Health and Exercise Science department at the University of Oklahoma and the OUHSC Institutional Review Board.

What Are the Costs?

There is no cost to you for participating in this study.

Will I Be Paid For Participating in This Study?

All individuals will be compensated for their time commitment associated with the study. Participants will be paid $50 for each visit, and a total of $200.

What if I am Injured or Become Ill While Participating in this Study?

In the case of injury or illness resulting from this study, emergency medical treatment will be available. If injury occurs as a result of participation, you should consult with your personal physician to obtain treatment. No funds, however, have been set aside by The University of Oklahoma Health Sciences Center or University of Oklahoma to compensate you or pay for the costs associated with treatment in the event of injury.

What Are My Rights As a Participant?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. If you agree to take part and then decide against it, you can withdraw for any reason, so please be sure to discuss leaving the study with the principal investigator or your regular physician. Refusal to participate will not result in any penalty or loss of benefits that you are otherwise entitled.

We will tell you about any significant new findings developed during the course of the research that may affect your health, welfare or willingness to stay in this study.

You have the right to request the medical information that has been collected about you as a part of this research study. At this point, you will not have access to the biomedical related information that will be collected from you during this study and you consent to this restriction.

For questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

Whom Do I Call If I have Questions or Problems?

If you have questions, concerns, or complaints about the study or have a research-related injury, contact Chad Kerksick, PhD at 405-325-9021 (office) or 405-248-8730 (cell).

If you cannot reach the Investigator or wish to speak to someone other than the investigator, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

For questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

Signature:

By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

Research Subject: _____

Date:_____

Subject's Printed Name: _____

Person Obtaining Informed Consent:_____

Date:_____

IRB Office Version Date: 11/01/06

Appendix E

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University of Oklahoma

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Impact of Age on Androgen Receptor Expression and Binding Affinity after Sequential Bouts of Lower Body Resistance Exercise

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General Health and History Form

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Side A

Demographics:

Name: _____   Subject number: _____

Date: _____   Age: _____   Birth Date: _____

Daytime phone: _____   Evening contact number: _____

Family History:

Has anyone in your immediate family had any of the following: Please circle **yes** or **no**

Heart disease	Yes	No	Diabetes	Yes	No
High blood pressure	Yes	No	Cancer	Yes	No
Stroke	Yes	No	Tuberculosis	Yes	No
Sudden Death (before 50)	Yes	No	Asthma	Yes	No
Epilepsy	Yes	No	Gout	Yes	No
Migraine Headaches	Yes	No	Marfan's Syndrome	Yes	No
Eating Disorder	Yes	No	Sickle Cell	Yes	No

Personal History:

1. Have you ever been hospitalized?   Yes   No

Have you ever had surgery?   Yes   No

Are you presently under a doctor's care?   Yes   No

Please explain and give dates for all "Yes" answers: _____

2. Please list any medications you are currently taking and for what conditions: _____

3. Please list any known allergies: _____

4. Have you ever had a head injury / concussion?   Yes   No

Have you ever been knocked out or unconscious?   Yes   No

Have you ever had a seizure, "fit", or epilepsy?   Yes   No

Have you ever had a stinger, burner, or pinched nerve?   Yes   No

Do you have recurring headaches or migraines?   Yes   No

Please explain and give dates of "yes" answers: _____

5. Have you ever had the chicken pox?   Yes   No

     If Yes, at what age? _____

6. Have you ever had the mumps pr measles?   Yes   No

7. Do you have a history of asthma?   Yes   No

8. Are you missing an eye, kidney, lung, or testicle?   Yes   No

9. Do you have any problems with your eyes or vision?   Yes   No

10. Have you ever had any other medical problems   Yes   No
(mononucleosis, diabetes, anemia)?

11. Have you ever taken any supplements for improved performance?   Yes   No

12. Are you presently taking any supplements for diet or performance   Yes   No
(creatine, protein, etc.)?

University of Oklahoma

Impact of Age on Androgen Receptor Expression and Binding Affinity after Sequential Bouts of Lower Body Resistance Exercise

General Health and History Form

Side B

13. What is the lowest weight you have been at in the last year _____,
Highest _____, What is your ideal weight _____?

14. Do you have any trouble breathing or do you cough during or after practice?
      Yes   No

15. Have you ever had heat cramps, heat illness, or muscle cramps?   Yes   No

16. Do you have any skin problems (itching, rashes, acne)?   Yes   No

Explain all "Yes" answers for question 5 -16: _____

17. Have you ever passed out during or after exercise?   Yes   No
Have you ever been dizzy during or after exercise?   Yes   No
Have you ever had chest pain during or after exercise?   Yes   No
Have you ever had high blood pressure?   Yes   No
Have you ever been told you have a heart murmur?   Yes   No
Have you ever had racing of your heart or a skipped heartbeat?   Yes   No
Have you ever had an EKG or echocardiogram?   Yes   No

Explain all "Yes" answers for question 17: _____

18. Have you ever sprained / strained, dislocated, fractured, or had repeated
swelling or other
injury of any bones or joints? Explain any "Yes" answers

Head / Neck	Yes	No	_____
Shoulder	Yes	No	_____
Elbow & Arm	Yes	No	_____
Wrist, hand & Fingers	Yes	No	_____
Back	Yes	No	_____
Hip / Thigh	Yes	No	_____
Knee	Yes	No	_____
Shin / Calf	Yes	No	_____
Ankle, foot, toes	Yes	No	_____

Please Sign:

I hereby state that, to the best of my knowledge, my answers to the above questions are correct.

Subject's Signature: _____ Date: _____

Appendix F

Department of Health and Exercise Science - University of Oklahoma-Norman Campus

Effects of repeated bouts of weight-training on androgen receptor expression in skeletal muscle of young versus old participants

To the Attending Physician of: _____

This individual has indicated that he wishes to participate in a research study investigating the effects of resistance training of intramuscular markers of muscle growth in younger and older men. The outcomes of this study will help researchers further delineate how exercise can combat muscle aging. This project has been approved by the Institutional Review Board at the University of Oklahoma.

Description of the Study
This study will consist of 7 visits to the laboratory. During this first visit, all participants will have their maximal strength determined in their lower extremity muscles (see exercises below in the "Resistance training" sub-section). Before this strength determination blood pressure will be obtained a small sample of muscle tissue from the thigh region will be collected. In short, this muscle collection involves the use of local anesthesia (2% Lidocaine) and a 5-mm Bergstrom biopsy needle. Our lab has previously performed these specimen collection procedures in hundreds of participants and no one to date has reported any deleterious side-effects.

Resistance training
The strength assessment and workouts consist of lower body resistance exercises using a modified squat (minimally stressing the lower back), leg press, and a leg extension machine.

Visits 2 and 3
Participants will perform 2 sets of 10 repetitions of each of the 3 exercises including a modified squat (minimally stressing the lower back), leg press, and leg extension exercise. These visits are considered to be practice exercise bouts to familiarize the participant with resistance training.

Visits 4, 5, 6 and 7
Participants will report to the lab on Mon (visit 4), Wed (visit 5), Fri (visit 6), and Sat (visit 7). During visits 4, 5, and 6 the participant will perform 3 sets of 10 repetitions of each of the 3 exercises including a modified squat (minimally stressing the lower back), leg press, and leg extension exercise. Muscle will be collected prior to exercise (on visits 5, 6) and blood only will be collected after exercise (on visits 4, 5, 6). Blood and muscle will be collected on the last visit (visit 7) which involves no exercise.

Please advise the investigators regarding any physical limitations and/or contraindications that this patient might have from engaging in this exercise study.

Pertaining to the above mentioned patient, I advise the following:

☐   To my knowledge, there is no reason why this patient should not be allowed to participate in this study.

☐   I recommend that this patient be allowed to participate in the study with the following restrictions:_____

☐   I recommend that this patient should **not** be allowed to participate in the study for the following reasons:_____

Physician's Name _____Date_____

If you have any questions about this form, please contact: Chad Kerksick, Ph.D., Assistant Professor, Director, Applied Biochemistry and Molecular Physiology Laboratory at 405-325-9021