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THE EFFECTS OF AGING ON MARKERS OF SATELLITE CELL ACTIVITY AFTER THREE SEQUENTIAL BOUTS OF RESISTANCE EXERCISE

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THE EFFECTS OF AGING ON MARKERS OF SATELLITE CELL ACTIVITY AFTER THREE SEQUENTIAL BOUTS OF RESISTANCE EXERCISE

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

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"The road to Easy Street goes through the sewer."

~John Madden

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ABSTRACT

THE EFFECTS OF AGING ON MARKERS OF SATELLITE CELL ACTIVITY AFTER THREE SEQUENTIAL BOUTS OF RESISTANCE EXERCISE

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Background. Satellite cell content in human skeletal muscle has shown to be reduced in older versus younger populations. Further, the ability of older satellite cells to proliferate following resistance exercise has been shown to be impaired in older individuals; an effect which seemingly limits the hypertrophic response to resistance exercise with aging. Previous *in vitro* and *in vivo* research has demonstrated that genes involved in satellite cell proliferation, myoblast differentiation, and insulin-like growth factor (IGF)-1 signaling are differentially expressed in older versus younger muscle. However, no evidence to date exists examining how multiple bouts of resistance training affect the genetic and proteomic expression of these indices between younger and older age groups.

Purpose. Therefore, the purposes of this study were to: 1) assess baseline markers indicative of satellite cell activity between younger versus older males, 2) examine if three sequential bouts of resistance exercise affects markers of satellite cell activity in

these populations, and 3) examine if the potential age-dependent decrements in satellite cell proliferation and/or differentiation were related to decrements in myogenic genes and/or a decrement in muscle IGF-1 protein expression in older individuals at baseline and throughout the resistance training intervention.

Methods. Ten younger (age: 18-25 y) and 10 older (age: 60-75 y) males were recruited for this study and were instructed to complete 3 lower body workouts (M, W, F) consisting of 9 sets of lower-body exercises with 10 repetitions per set at an intensity of 80% of each individual's one repetition maximum. Percutaneous muscle biopsies were collected 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). A fraction (~20 mg) of muscle tissue was rinsed of excess blood, connective, and adipose tissue and was homogenized in order to assess the concentrations (denoted by [__]) of muscle [DNA], [total protein], and [myofibrillar protein]. A second fraction of muscle (~30 mg) was also homogenized, total RNA was precipitated out the homogenate and mRNA from the total RNA pool was reverse transcribed into cDNA for gene expression analysis. Finally, a third fraction of muscle (~25 mg) was pre-rinsed and homogenized to obtain total protein for subsequent Western blotting. Semi-quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed to quantitate baseline and changes in the mRNA expression levels of *CYCLIN D1*, cyclin dependent kinase *(CDK)4*, *CDK2*, $P21^{CIP1}$, $P27^{KIP1}$, *MYOD*, *MGF*, *IGF-IEA*, and embryonic myosin heavy chain (MHC_{EMB}) with the arithmetic mean of beta-2 microglobulin (*B2M*) and 28S rRNA (*28S*) being used as an internal control. Muscle homogenates were also assayed for the protein contents of

proliferating cell nuclear antigen (PCNA), hepatic growth factor receptor (c-met), and muscle IGF-1.

Results. In partial agreement with my initial hypotheses, repeated bouts of conventional resistance training increased muscle [c-met], a protein marker indicative of satellite cell quantity, in young males 48 hours following the first bout ($p < 0.01$) and decreased this marker in young males 24 hours following the third bout ($p < 0.05$), whereas this value did not change within the old group. Furthermore, *MYOD* gene expression modestly increased at T2 ($p < 0.05$) and MHC_{EMB} gene expression modestly increased ($p < 0.05$) at T4 relative to baseline expression values in the younger males. Interestingly, *MGF* expression increased at T2-4 in the older group relative to baseline values ($p < 0.05$), albeit muscle IGF-1 peptide levels remained stable throughout the intervention in both age groups. Other findings from this study indicate that repeated training bouts: 1) do not alter the expression of genes indicative of satellite cell activity (i.e., *CDK2*, *CDK4*, *CYCLIN D1*, *MYOD*, $P27^{KIP1}$, $P21^{CIP1}$) and/or the expression of *IGF-1EA* up to 24-48 hours following exercise in younger or older males, 2) do not increase markers of satellite cell proliferation (i.e., muscle [PCNA], and [DNA]) in either age group at the sampled time points, and 3) do not alter [myofibrillar protein] or [total protein] in either age group at the sampled time points. In regards to baseline physiological parameters, these data illustrate that the *CYCLIN D1* gene is expressed more highly in older versus younger males ($p < 0.05$), whereas all other genes and markers of satellite cell activity were similar between age groups.

Conclusions. The findings from this study suggest: 1) both age groups retain proliferative characteristics during resting, unexercised states given the similarities

between these groups in regards to muscle [PCNA], muscle [c-met], *CDK2*, *CDK4*, as well as an increased *CYCLIN D1* mRNA expression in older individuals, 2) three consecutive bouts of resistance exercise seem to significantly increase a marker indicative of satellite cell quantity concomitant with the increased expression of a select few myogenic genes in younger participants only (i.e., *MYOD* at T2 and MHC_{EMB} at T4), and 3) repeated exercise bouts facilitated a summation effect on *MGF* expression only in older individuals which is contrary to preliminary research examining this gene in these populations. In summary, these data indicate that more exercise bouts may be needed to stimulate satellite cell activity during the initial stages of resistance training in older populations which possibly explains why there are impairments in muscle hypertrophy in older adults, albeit future research should employ immunohistochemistry in order to confirm these preliminary findings.

CHAPTER I

INTRODUCTION

Sarcopenia (Greek translation meaning "*poverty of the flesh*") describes the gradual but inevitable decline in muscle mass and strength that accompanies biological aging. Sarcopenia is associated with a sundry of intramuscular events including: 1) a reduction in the size and number of fast-twitch (i.e., type II) muscle fibers [1], type II motor units [2], and type II fiber satellite cells [3], 2) a reduction of myonuclei in post-mitotic myofibers [4], and 3) decrements in contractile protein synthesis [5], and 4) increases in intramuscular proteolysis [6]. Furthermore, other environmental or exogenous cues including: 1) decreases in physical activity [7], 2) inadequate dietary protein consumption [8], 3) decrements in endocrinological factors [i.e., bioavailable testosterone in males and insulin-like growth factor (IGF)-1 in both sexes [9]], and 4) decreases in the production of myogenic autocrine/paracrine factors (i.e., IGF-IEa and mechano growth factor (MGF) variants [10]) also accompany and/or exacerbate sarcopenia. While the primary cause of sarcopenia remains elusive, the phenotypic manifestation of all of the aforementioned events includes an estimated 20-30% decrease in lean body mass between the third and eighth decades of life [11].

Resistance exercise can prevent and even reverse the progression of sarcopenia [9, 12]. Furthermore, resistance exercise has been demonstrated to acutely increase muscle protein synthesis and translational efficiency [13], acutely increase markers of satellite cell proliferation, and chronically increase satellite cell number [12] and myonuclei number per myofiber [14] which is suggestive of myoblast differentiation and fusion events. However, there is histological and body composition evidence suggesting that

hypertrophic adaptations following months of structured exercise are limited in older versus younger populations [9, 15]. For instance, Petrella et al. [14] have suggested that the pre-existing number of satellite cells determines the hypertrophic potential to a resistance training regimen. Likewise, Verdijk et al. [12] have recently discovered that the number of satellite cells residing next to type II fibers is limited in older versus younger individuals. Finally, sparse evidence exists suggesting that the proliferative capacity of satellite cells [16] as well as the molecular pathways which regulate satellite cell activity is dysregulated in older humans [17, 18] and mammals [19]. Taking all of this information into consideration, it seems plausible that a dysfunction in satellite cell activity with aging exacerbates losses in lean tissue mass.

The involvement of IGF-1 signaling with satellite cell activity is of particular interest due to that fact that: 1) IGF-1 variants [i.e., mechano growth factor *(MGF)* and *IGF-1EA*] are locally expressed in skeletal muscle following mechanical loading [10, 20], and 2) these variants have been shown to possess a causal role in inducing quiescent satellite cells to proliferate and differentiate [21]. Interestingly, past literature has determined that older rodents and humans exhibit a decrement in *MGF* and/or *IGF-1EA* mRNA expression following an acute bout of exercise [10, 15, 22], and limited human evidence [15, 23] has suggested that the acute post-exercise decrease in *MGF/IGF-1EA* mRNA expression is associated with a long-term decrement in phenotypic adaptations (i.e., increases in muscle size) accrued from resistance training. However, no evidence exists in humans demonstrating that the potential age-related decreases in *MGF/IGF-1EA* mRNA and protein expression patterns following a series of resistance exercise bouts are linked to a reduction in satellite cell activity. Therefore, the purposes of this study are to:

1) assess baseline markers indicative of satellite cell proliferation and differentiation between younger versus older males, 2) examine if three sequential bouts of resistance exercise affects markers of satellite cell proliferation and/or differentiation in both age groups, and 3) examine if the potential age-dependent decreases in satellite cell proliferation and/or differentiation are related to decrements in muscle *MGF/IGF-1EA* mRNA and IGF-1 protein expression in older individuals. If there are genes (i.e., *MGF, IGF-1EA* or other cell cycle regulators examined in the current study) that are dysregulated between age groups and are linked to decrements in satellite cell activity, then the findings from this study will warrant future research in determining the potential epigenetic modifications (i.e., genes that are permanently "turned off") that may occur in aging satellite cells and/or post mitotic muscle fibers. Such findings would heavily support the notion of employing gene therapy as a future modality to treat sarcopenic populations. In this regard, transplanting genetically modified satellite cells, which possess the transcriptomic pattern of younger satellite cells, into the skeletal muscle bed of sarcopenic populations could be employed; this being a method which has already been successfully performed with *mdx* mice [24].

Hypotheses

1. It is hypothesized that baseline (or pre-exercise intervention) markers indicative of satellite cell proliferation (i.e., hepatocyte growth factor receptor (c-met) protein, proliferating cell nuclear antigen (PCNA) protein, [DNA], and the mRNA expression of *CYCLIN D1*, cyclin dependent kinase (*CDK*)*4* and *CDK2*) as well as embryonic myosin heavy chain (MHC_{EMB}) mRNA expression, myofibrillar

protein content, and muscle *MGF/IGF-1EA* mRNA and IGF-1 protein will be greater in younger participants whereas baseline expression of genes indicative of terminal differentiation (i.e., *MYOD, P21^{CIP1}, P27^{KIP1}*) will be greater in older participants.

2. It is hypothesized that three sequential bouts of lower-body resistance exercise will continually increase markers of satellite cell proliferation and terminal differentiation in both age groups following each exercise bout, albeit increases in all of these markers will be diminished in older versus younger males.
3. It is hypothesized that older participants will express less muscle *MGF*/*IGF-1EA* mRNA and IGF-1 protein, and the lack of this IGF-1 "signal" will be associated with the attenuation in the aforementioned markers of satellite cell activity. For instance, it is likely that there will be negative correlations between the delta changes in *MGF/IGF-1EA* mRNA or IGF-1 protein expression patterns and the delta changes in markers of satellite cell activity.

Definitions of Terms and Procedures

Satellite cells – constitute a majority of muscle precursor cells and reside between the sarcolemma of mature muscle fibers and the basement membrane (i.e., connective tissue rich in laminin and collagen proteins)

Quiescence – also referred to as the G_0 phase; a state of cellular dormancy prior to interphase or mitosis

Senescence – a state of post mitosis whereby cells can no longer divide

Mitogen – a protein or other signal that triggers cellular division (i.e., mitosis)

Proliferation – also referred to as the G_1, S, and G_2 phases of the cell cycle (which can also be termed "interphase") as well as the M phase; the process of DNA replication and subsequent cellular division to increase the number of resident precursor cells

Differentiation – the process of precursor cells (i.e., satellite cells) ceasing proliferation and becoming a more specialized cell type (i.e., a myofiber with a centralized nuclei and sparse organellular content compared to mature myofibers); differentiation is signified in part by the expression of muscle specific genes including myosin heavy chain isoforms, creatine kinase, and other gene species

Myogenic regulatory factors – are transcription factors (i.e., MyoD, myogenin, MRF4, Myf5) that belong to the basic helix-loop-helix protein family; in general these proteins form active heterodimers with ubiquitously expressed E proteins which subsequently bind to the upstream promoter regions (i.e., E boxes) of genes related to differentiation thereby increasing transcription rates

Myonuclear domain – this is the volume of cytoplasm that is "governed" by a myonucleus [reported as 20,000 – 30,000 μm^3 [25] across all fiber types]; as a fiber grows, more myonuclei must be recruited to conjoin to the cell to maintain cellular function [25]

Kilodalton – abbreviated as kDa; 1 kDa = 1,000 Daltons (Da) where 1 Da is the mass of a hydrogen atom; kDa masses are used to describe proteins due to their large molecular weights

C-met – a membrane-bound tyrosine kinase receptor (molecular weights: α-chain = 50 kDa, β-chain = 145 kDa) whose ligand is hepatocyte growth factor; in skeletal muscle tissue, these receptors are solely expressed in satellite cells and proliferating myoblasts

Retinoblastoma 1 – This protein (molecular weight: 110 kDa) belongs to the pocket protein family and is a nuclear protein that inhibits the progression of the cell cycle [26]

Cyclins – are proteins that are expressed in a cyclical fashion during the cell cycle whereby they bind to and activate cdks; the cyclin of interest in this experiment include cyclin D1 (G_1 phase cyclin) due to its induction by MGF/IGF-1Ea [27]

Cyclin dependent kinases – abbreviated as cdks; these enzymes are a group of protein kinases that regulate the cell cycle and are active when bound to cyclins [26]

Cdk-inhibitors – are proteins that inhibit cdks by binding to cdk-cyclin complexes [26]; the cdk-inhibitors of interest in this investigation include $p21^{Cip1}$ and $p27^{Kip1}$ due to the fact that they are obligatory for satellite cell cycle exit and differentiation [28], they are exercise inducible [29], and they are differentially expressed between age groups [19, 30]

Myofibrillar protein – is protein that makes up myofibrils; for example, some of these proteins include the various isoforms of myosin, actin, troponin, titin, and nebulin

Total muscle protein – includes all membrane-associated proteins (i.e., receptors and cytoskeletal proteins), cytoplasmic proteins (i.e., metabolic enzymes, nuclear receptors, etc), myofibrillar proteins, and nuclear proteins (i.e., transcription factors)

Embryonic myosin heavy chain – abbreviated as MHC_{emb}; this myosin isoform has been shown to be expressed in very small regenerating fibers and is thought to signify the presence of newly differentiated myotubes that fuse to pre-existing muscle fibers [31]

DNA – deoxyribonucleic acid is a double-stranded molecule that makes up chromatin/chromosomes which contain genetic information that is able to be transcribed into mRNA messages and translated (via polyribosomes) into functional proteins

Total RNA – ribonucleic acid are single-stranded molecules that include three subtypes: 1) ribosomal RNA (or rRNA including the 18S, 5.8S, 28S and 5S subtypes) make up ~80% of the total RNA pool which are thousand nucleotide length molecules and, along with ribosomal proteins, make up the 80S ribosome which carries out protein synthesis; 2) transfer RNA (or tRNA) make up ~15% of the total RNA pool and are 70-90 nucleotide length RNA molecules that transfer amino acids to growing polypeptide chains during protein synthesis; 3) messenger RNA (or mRNA) make up ~5% of the total RNA pool which are hundred nucleotide length molecules that carry genetic information from genes to ribosomes

Translational efficiency – the rate of mRNA translation into proteins; if translational efficiency increases then rates of translation are enhanced without increases in the number of 80S ribosomes; an effect which is primarily due to an increase in translation initiation [32]

Translational capacity – The number of ribosomes which are available to translate mRNA transcripts; translational capacity increases in cells after growth factor stimulation via the phosphorylation of ribosomal protein S6 and the subsequent preferential translation of ribosomal protein mRNAs; after new ribosomes are formed (i.e., translational capacity increases) then other mRNAs (i.e., cell cycle regulator mRNAs) are preferentially translated [33]

Muscle homogenation – refers to the process of using various buffers to solubilize muscle into a 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 that contains chemicals (i.e., phenol and guanidine thiocyanate) to catalyze an immediate inhibition of RNase activity; samples are: 1) first homogenized or lysed in Tri Reagent and the homogenate is separated into aqueous and organic phases by chloroform addition and centrifugation, 2) RNA in the upper aqueous phase is then precipitated by the addition of isopropanol, washed with ethanol and solubilized with RNase-free water for subsequent gene expression analysis

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, 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

Ponceau S staining – this procedure is used to mathematically correct Western blotting values for lane-to-lane loading variations

Real time reverse transcriptase polymerase chain reaction – a multi-step procedure which uses gene-specific primer sequences and a fluorescent tracer to detect the expression of explicit mRNA transcripts (termed genes of interests or GOIs)

Housekeeping gene – a gene that is used to normalize GOI expression in order to correct for well-to-well loading variations between samples

Melt Curve – a *post hoc* procedure used to confirm the presence of one cDNA amplification product (or gene) during PCR; if multiple peaks are present in a given well following melt curve analysis then this signifies the presence of other gene products (i.e., splice variants, single nucleotide polymorphisms, primer-dimers, or contaminant DNA)

Hoechst staining – is a method discriminatingly detects double-stranded nucleotides (i.e., DNA) in the presence of RNA and other nucleotides by the binding of Hoechst dye to AT-rich sequences in the minor groove of DNA; this method can be performed on crude muscle homogenates (i.e., no multi-step phase separation of DNA is required) to determine if DNA replication has occurred [22]

One repetition maximum – the maximum amount of weight a participant is able to eccentrically and concentrically perform on a given exercise apparatus

Abbreviations

Cdk – cyclin-dependent kinase

$p21^{Cip1}$ – cyclin-dependent kinase inhibitor 1A

$p27^{Kip1}$ – cyclin-dependent kinase inhibitor 1B

pRb – retinoblastoma 1

PCNA – proliferating cell nuclear antigen

MyoD – myogenic differentiation

MHC_{emb} – embryonic myosin heavy chain

RNA – ribonucleic acid

DNA – deoxyribonucleic acid

IGF – insulin-like growth factor

MGF – mechano growth factor

HGF – hepatocyte growth factor

MRF – myogenic regulatory factor

RT-PCR – reverse transcriptase polymerase chain reaction

cDNA – copy DNA

GOI – gene of interest

[__] – signifies concentration; for instance, muscle [DNA] signifies muscle DNA concentration

Delimitations

Ten older (60-75 y) and ten younger (18-25 y) males who were not currently participating in any form of resistance training with their lower bodies were recruited for this study. Prior to data collection, all participants completed a written statement of informed consent and medical history questionnaire. Eligibility inclusion criteria included the following: 1) apparently healthy participants that did not participate in a structured lower-body resistance exercise regimen consistently (i.e., at least one time per week) one year prior to participation in this study, 2) participants were told to refrain from smoking, alcohol, tobacco or caffeine for the duration of the study, 3) participants could not have consumed ergogenic nutritional supplements for at least 3 months prior to the start of the study, 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 any orthopedic problems (e.g., previous surgery, joint replacements, etc.) or previously diagnosed neuromuscular disease that prevented them from participating in the resistance training sessions, 7) participants could not be taking prescription medications indicated for heart, pulmonary, anti-coagulant, anti-hypertensive, psychotropic, neuromuscular/neurological, or androgenic dysfunctions, 8) participants could not have any absolute or relative

contraindication for exercise testing as outlined by the American College of Sports Medicine (provided below):

Absolute Contraindications to Exercise Testing

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

Eligibility inclusion criteria list continued: 9) Participants could not have an allergy to Lidocaine, 10) Participants could not have an allergy to latex, or 11) Older participants that did not provide written medical clearance from their primary physician.

Assumptions

1. Participants filled out medical history questionnaires correctly.
2. Participants followed the guidelines established by the investigators throughout the duration of the study (i.e., participants refrained from consuming supplements, tobacco, 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 study were representative of younger and older male populations.

Limitations

Theoretical limitations

1. Younger and older participants were recruited from the University of Oklahoma campus and younger participants were recruited within the Department of Health and

Exercise Science. Furthermore, participants received a $150 incentive for their time and effort throughout the duration of the study. These two facets theoretically violate the idea of random subject selection.

Methodological limitations

1. Muscle [DNA] determination: using Hoechst staining, delicate increases in DNA caused by increases in DNA synthesis from satellite cell activation and myoblast proliferation will be able to be detected. This method has been successfully employed by Haddad and Adams [22] to determine the age-related differences in DNA content within the skeletal muscle of rats. The predominant limitation to this method, as proposed by Booth [34], includes quantifying DNA from other cells in the muscle bed including stromal vascular cell lines (i.e., fibroblasts, smooth muscle, epithelial cells) infiltrating leukocytes, intramuscular adipocytes as well as DNA from pre-existing muscle fibers. To minimize this interference, muscle samples will be blotted dry on sterile absorbent tissue and dissecting tools were used to cleanse each sample of excess adipose tissue and connective tissue. Furthermore, muscle homogenates from each sample were immunoprobed for a marker (CD163) exclusively expressed by monocytes/macrophages that potentially enters the muscle tissue following each exercise bout.
2. Interpreting changes in [myofibrillar protein]: the addition of myofibrils can arise from: 1) satellite cell differentiation and fusion to pre-existing myofibers and/or, 2) an increase in myofibrillar protein synthesis in pre-existing muscle fibers. Thus, [myofibrillar protein] was expressed as raw data and was corrected for [DNA] in order to infer what could be hypothetically occurring. For instance, if [myofibrillar

protein]: [DNA] increased then this signified an increase in myofibrillar protein synthesis in the absence of satellite cell proliferation/differentiation/fusion. Conversely, if [myofibrillar protein] and [DNA] increased, but the [myofibrillar protein]: [DNA] remained statistically unaltered then this (along with concomitant increases in MRF, cdk-inhibitor, and MHC_{EMB} mRNA expression patterns) strongly suggested that satellite cell proliferation/differentiation/fusion to pre-existing fibers may have likely occurred.

CHAPTER II

REVIEW OF LITERATURE

The Findings of Alexander Mauro (1961): a Historical Perspective

Satellite cells are mononuclear muscle precursor cells that lie between the sarcolemma of mature muscle fibers and the basal lamina, or connective tissue in the extracellular matrix. These "resident stem cells" have been under intense investigation since their discovery in frog muscle by Alexander Mauro [35]. Furthermore, Mauro's findings have led to heated debates as to whether satellite cells are even needed for exercise-induced muscle hypertrophy to occur. In this regard, a wealth of evidence has demonstrated that chronic mechanical loading in rodents and humans induces an increase in satellite cell number and an eventual increase in post-mitotic fiber nuclei; a phenomenon which is thought to occur as a result of the fusion of differentiated, mononucleated satellite cells to pre-existing fibers [36]. Likewise, inducing the necrosis of satellite cells with radiation prior to exercise training has been shown to completely abolish muscle growth in rodents further demonstrating their integral role in exercise-induced hypertrophy [37]. However, a select group of scientists (in particular Drs. Esser and McCarthy) have provided a unique perspective (supported by sparse evidence) that satellite cells assume a passive role in skeletal muscle physiology and that muscle growth is chiefly due to an increase in the post-mitotic myonuclear expression and translation of myofibrillar proteins followed by their subsequent incorporation into pre-existing myofibrils [38]. When integrating these two perspectives, it seems plausible that the acute-phase hypertrophic response to resistance exercise involves the post-mitotic myonuclear expression and translation of myofibrillar proteins followed by their

subsequent incorporation into pre-existing myofibrils, whereas the chronic hypertrophic response to a series of exercise stimuli is mediated by the addition (fusion) of differentiated satellite cells to pre-existing muscle fibers [39].

Upon his discoveries, Mauro devised three hypotheses as to how satellite cells are fabricated. First (Figure 1A), Mauro speculated that the myonuclei of a damaged muscle fiber "*gathers up*" cytoplasm from the damaged multinucleated cell in order to spawn a new, undamaged satellite cell that is able to regenerate a new muscle cell. This hypothesis was, however, deemed "*an unusual mechanism for vertebrate systems*" by Mauro given that sparse evidence supported this model. The second hypothesis coined by Mauro (Figure 1B) was that satellite cells were "*remnants of embryonic development*" that remained in the muscle bed following the fusion of mononucleated myoblasts and subsequent formation of multinucleated myotubes. Mauro goes on to say that these remaining reservoirs of unincorporated satellite cells "…are *ready to recapitulate the embryonic development of skeletal muscle fiber when the main multinucleate cell is damaged.*" Finally, Mauro's third hypothesis (Figure 1C) was that satellite cells were "*wandering cells*" that infiltrate the muscle bed and await the proper conditions to be activated.

The recent advent of more discriminatory microscopic techniques allowed scientists to confirm that Mauro's second hypothesis (Figure 1B) was the most apparent explanation of the origin of satellite cells. For example, Schienda et al. [40] used reporter protein methods (i.e., infecting chick somites with a retrovirus fused the green fluorescent protein reporter) to label and routinely examine the morphogenesis of mammalian somitic cells during embryonic development using immunofluorescent microscopy. In

short, these authors concluded that somitic cells, which are embryonic cells lateral to the embryonic neural tube that eventually give rise to skeletal muscle, are also the origin of satellite cells. It is interesting to note, however, that Mauro's third hypothesis (Figure 1C) may also occur due to the presence of heterogeneously phenotypic satellite cells. For instance, Rantanen et al. [41] discovered that a subpopulation of satellite cells exist in the muscle bed which are able to express myogenin and commit to differentiation without proliferation within 4-8 hours of muscle damage, whereas another population of "stem satellite cells" also exist and do not divide until 24 hours following muscle damage. To summarize their findings, these authors stated:

> "*…we propose that there are two populations of precursor cells: committed satellite cells, which are ready for immediate differentiation without preceding cell division, and stem satellite cells, which undergo mitosis before providing one daughter cell for differentiation and another for future proliferation.*"

In an attempt to explain this phenotypical divergence, Zammit et al. [42] authored an extensive review that provides a wealth of evidence to suggest other multipotent stem cells including interstitial cells, endothelial-associated cells, and side population cells (i.e., bone marrow-derived hematopoietic stem cells) share similar molecular signatures (or express several of the same proteins) and are able to adopt the myogenic lineage following transplantation experiments. Regardless, Zammit and colleagues also admit that scientists have been relatively unsuccessful in determining whether or not "*wandering stem cells*" meaningfully contribute to the satellite cell reservoir. Further, these authors conclude that the heterogeneous phenotypic activity of satellite cells may be a result of these cells existing in a "*dynamic system*".

Overview of the Cell Cycle

According to historical literature, the eukaryotic cell cycle has been a subject of intense interest for physiologists since the microscopic examination of the nematode (*Trichinella spiralis*) life cycle by German scientist Rudolf Virchow in the 1850's [43]. The cell cycle is comprised of several distinct phases which have been extensively reviewed [44-48] and will be briefly described in this section. During the first phase (G_1, or Gap 1) the cell body grows and there is a concomitant increase in the production of enzymes that are required for DNA replication. Once the cell has attained a certain size it enters the S (synthesis) phase, whereby the cell duplicates each chromosome. The cell then enters the G_2 (Gap 2) phase whereby a significant amount of protein synthesis occurs; mainly involving the production of microtubules which make up mitotic spindles that are obligatory for mitosis. Finally, duplicated chromosomes are separated during the M (mitosis) phase and the cell subsequently divides (i.e., cytokinesis) spawning the formation of two genetically identical daughter cells. After mitosis is complete, the two daughter cells can continue to replicate (i.e., re-enter the G_1 phase), or they can exit from the cell cycle and enter a mitotically quiescent stage (G_0) until it is again activated by a mitogenic stimulus. In most mammalian cells, the entire cell cycle has been observed to last between 10 and 30 hours [48].

While mechanisms of the cell cycle remained speculative during the subsequent decades following Virchow's nematode studies, the endeavors of geneticists Drs. Leland Hartwell, Paul Nurse, and Timothy Hunt during the 1970's and 1980's revealed explicit genes, which were later termed "cyclin dependent kinases" at the Cold Harbor Symposium on Cell Cycle in 1991, that controlled various checkpoints of the cell cycle

[26]. Cdk proteins possess serine/threonine kinase domain, although scientists determined that these enzymes must to bind to cyclin proteins subsequently be phosphorylated by a cdk-activating kinase in order to exert their biochemical effects [26]. Furthermore, specific active cdk-cyclin complexes are typically localized or sequestered into the nucleus during different phases of the cell cycle where they act to phosphorylate downstream targets. For instance, immunofluorescence microscopy has been used to localize cyclin A in the nuclei of proliferating cells during and following the S phase, whereas cyclin B1 accumulates in these cells during the G_1, S and G_2 phases and is translocated into the nucleus during the M phase when it is predominantly active [49]. The functional ramifications of cdk-cyclin nuclear localization are evident given that the nuclear localization of cyclin A during the S phase (discussed in the sentence above) parallels observations to suggest that cdk2-cyclin A phosphorylates nuclear proteins involved in DNA replication, DNA repair, and histone modification [26].

It is noteworthy to mention that bioinformatic analysis has been used to identify 29 mammalian cyclins which possess a conserved cyclin box domain that act as binding templates for cdks [26]. Likewise, 20 cdk proteins have been identified which possess a conserved serine/threonine kinase domain. However, cdks and cyclins of particular interest (i.e., those firmly linked to different phases of the cell cycle as well as those that are affected with exercise and aging in human skeletal muscle) will be discussed in subsequent paragraphs.

Satellite Cell Physiology and Molecular Markers of Satellite Cell Activation, Proliferation, Differentiation, and Fusion

As mentioned previously, satellite cells are mononuclear muscle precursor cells that reside between the sarcolemma of mature muscle fibers and the basal lamina of the extracellular matrix. A prominent molecular feature of satellite cells includes the co-expression of the paired box (*PAX*)*3/7* transcripts which encode transcription factors that: 1) commit these cells to the myogenic lineage [50], and 2) act in concert with other stimuli mentioned in subsequent paragraphs to induce the expression of MRFs during myogenesis [51]. Under normal physiological conditions, satellite cells remain in a non-proliferative, quiescent state (i.e., G_0). However, resident satellite cells can undergo sequential activation, proliferation, differentiation, and fusion events when skeletal muscle is exposed to trauma (i.e., experimental crushing or exposure to myotoxins), mechanical stress (i.e., overloading through synergist ablation or excessive stretching), or an adequate exercise stimulus [36]. The following paragraphs will describe these phenomena in greater detail as well as molecular markers that simultaneously appear during these events.

Divergent satellite cell populations. As mentioned previously, some researchers suggest that two distinct populations of satellite cells exist which display different phenotypic proliferative characteristics (depicted in Figure 2). In support of this hypothesis, Ambrioso et al. [36] suggest that rapidly (i.e., 4-8 hours) proliferating and differentiating satellite cells: 1) readily replicate thereby replenishing the satellite cells pool, and 2) are able to differentiate and fuse to pre-existing fibers in order to increase the number of myonuclei and maintain an ordinate myonuclear domain. Conversely, the prolonged (i.e., > 24 hours) self-renewing stem cells residing in muscle tissue, which may be of a different cellular origin as discussed above, are: 1) able to produce new muscle

stem cells, 2) are capable of differentiating without expressing markers of differentiation (i.e., MRFs), and 3) have the ability to differentiate into vascular or neural lineages following injury or extensive muscle tissue remodeling events. Interestingly, Booth's group [52] demonstrated that primary muscle progenitor cell populations isolated and cultured from rat skeletal muscle contain approximately 90% of "conventional" satellite cells (i.e., Pax7+, desmin+, MyoD+) whereas 10% of the isolated cells expressed hematopoietic stem cell markers (i.e., CD34+, CD45+) prior to passaging. Therefore, this evidence suggests that the addition of myonuclei to post-mitotic fibers plausibly occurs through the accretion of conventional satellite cells as well as satellite stem cells which possess different phenotypic characteristics.

Satellite cell activation. The binding of growth factors including IGF-1, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), nerve growth factor, leukemia inhibitory factor (LIF), and platelet-derived growth factor (PDGF) to cognate receptors present on the sarcolemma of satellite cells has been postulated to initiate satellite cell activation [36]. Once satellite cell activation occurs, a quiescent satellite cell enters the cell cycle (at this point, the cell is termed a proliferating myoblast) which is a multi-step process that yields two genetically identical daughter myoblasts from a single parent satellite cell. Quiescent satellite cells are morphologically flat, spindle-shaped cells with little cytoplasmic volume whereas myoblasts exhibit increases in cytoplasmic volume and organellular content [i.e., mitochondria, rough endoplasmic reticulum, and total RNA content which are needed for ATP production and protein synthesis during satellite cell growth and proliferation, respectively [53]]. Molecular markers that are indicative of satellite cell activation include the co-localization of HGF

with the c-met receptor as well as the expression of *c-fos* and *c-jun* which are deemed "early response genes" that act as transcription factors to increase the sequential expression of other genes needed for cellular proliferation. Other cell-specific markers that are used to microscopically quantitate satellite cell number include c-met, neural cell adhesion molecule (NCAM), and PCNA protein expression [15].

Interestingly, recent evidence suggests that physical activity transiently (i.e., 0-24 hours post-exercise) increases the myogenic (or muscle-borne) expression of a muscle-specific IGF-1variant [10, 20] and LIF [54] as well as the circulating appearance and possible extracellular liberation of HGF that is localized in muscle tissue in an inactive pro-peptide form [55]. Of particular relevance are the numerous investigations that have demonstrated that the myogenic expression of IGF-1 mRNA splice variants [i.e., liver-specific/systemic IGF-IEa and MGF] occurs in response to resistance exercise [10, 56, 57], eccentric contractions [58, 59], or functional overloading protocols in rodents [60]. Furthermore, recent evidence suggests that the transient myogenic expression of MGF following exercise acts to induce satellite cell activation and proliferation, whereas the prolonged myogenic expression of IGF-IEa following exercise (i.e., > 48 hours) acts to initiate myoblast differentiation [21]. Hence, the aforementioned literature suggests that exercise stimuli increase the expression and/or release of local growth factors (of interest IGF-1 variants) in post mitotic fibers which, in turn, act to initiate satellite cell activation (via MGF signaling), myoblast proliferation (via MGF signaling), and likely the differentiation myoblasts into myocytes (via IGF-IEa signaling) that are capable of fusing to pre-existing muscle fibers following exercise.

It is noteworthy to contemplate whether or not sarcolemmal and/or myofibrillar protein disruption, which commonly accompanies high-intensity eccentric muscle contractions, is obligatory for the subsequent occurrence of satellite cell activation [36]. In this regard, low-frequency, non-damaging electrical stimulation protocols in rodents [61] as well as chronic, low-intensity cycle ergometry training in humans [62] is capable of significantly increasing satellite cell activation and proliferation. Further, Mackey et al. [63] demonstrated that performing 3 weekly bouts of low intensity (15% 1RM) versus high intensity (70% 1RM) leg extensions induced a significantly greater increase in the number of active satellite cells in the former training group after 12 weeks of training; this being a peculiar finding due to the fact that greater weightlifting intensities, even when standardized for training volume, have been shown to elicit more pronounced skeletal muscle damage [64]. Interestingly, Bamman et al. [59] also reported that concentric versus eccentric squats elicited similar increases in myogenic *IGF-1* mRNA expression patterns 48 hours post-exercise (transcript variants were not discriminated) even though the eccentric training session elicited a more substantial increase in serum markers of myofiber damage. Finally, recent evidence exists demonstrating that a single, high volume/high intensity bout of eccentric leg extensions (i.e., a protocol that was meant to elicit myofibrillar disruption) substantially increased satellite cell proliferation in the absence of sarcolemma and/or myofibrillar protein disruption [65]. In summarizing their findings these authors stated,

> *"Despite 210 maximum voluntary contractions that were eccentric in nature being performed by the subjects in this study, seven of the eight subjects did not show myofibre lesions. Under light microscope examination, no disruption to the*

desmin or dystrophin proteins was found in the cellular membrane of the myofibres and, to support this, the fibronectin protein was not found within the myofibres. This indicated that the sarcolemma was undamaged. Even with the lack of myofibre necrosis, an increase in the number of satellite cells was confirmed by positive staining for NCAM, an abundant protein observed on the surface of early embryonic myotubes."

Therefore, while past research has demonstrated that satellite cell activity and myofiber damage synchronously increases following exercise, the aforementioned findings cumulatively suggest that myofibrillar damage accompanying eccentric muscle actions does not seem to be obligatory for satellite cell activation. Conversely, the resistance or endurance exercise-induced expression of IGF-1 variants from mature myofibers, note that evidence exists demonstrating that endurance training is also able to increase myogenic IGF-1 expression [66], as well as increases in the myonuclear domain following a series of exercise bouts (this mechanism being discussed in a subsequent paragraph) appear to be a candidate mechanisms that facilitate increases in satellite cell activity.

Satellite cell proliferation. The molecular signature of myoblast proliferation will be described in regards to each of the following phases of the cell cycle:

1) G_1 phase: this phase is marked by the transient expression of G_1 phase cyclins (cyclins D and E) and cdks (cdk4, cdk6, and cdk2) in satellite cells which act in tandem as catalytic cyclin-cdk heterodimers to hyperphosphorylate the retinoblastoma protein (pRb) in the satellite cell nucleus and facilitate DNA synthesis. Of particular importance, cdk4-cyclin D and cdk6-cyclin D purportedly act to initially phosphorylate and inactivate

pRb during the early portion of the G_1 phase, whereas cdk2-cyclin E continue an irreversible pRb phosphorylation process in the latter half of the G_1 phase [26]. It should be noted that hypophosphorylated pRb binds to and inhibits the activity of activator E2F transcription factors (E2F1, E2F2, and E2F3a) while recruiting histone deacetylases to chromatin which increases chromatin packing; these effects being deleterious to the transcription of genes necessary for DNA synthesis [67]. It should be noted that some but not all of the DNA synthesis genes whose expression is increased by activator E2F transcription factors include DNA polymerases α/δ (needed for leading and lagging strand synthesis) and thymidine kinase (needed for thymine nucleotide synthesis) [67]. It is also noteworthy that, at this point of the cell cycle, the expression and activity of MRFs (of interest myogenin and MyoD which initiate the differentiation process in proliferating myoblasts) have been reported to be lower than baseline levels if myoblasts continue proliferating or exit into quiescence following proliferation [27]. Conversely, MyoD expression can transiently peak and fall (via Ser200 phosphorylation and subsequent ubiquitin-mediated degradation) during the G_1 phase and peak again during the late G_2 phase in the subgroup of myoblasts that are induced to differentiate following the cell cycle; an effect which likely begins the differentiation process while proliferation is concomitantly occurring [68]. To summarize, an increased expression of G_1 phase cyclins and a decreased expression of MRFs are surrogate markers indicative of myoblast proliferation.

2) S phase: DNA synthesis, or the replication of all of the genetic material (i.e., chromatin) in a mitotically activated satellite cell to yield two copies of genetically identical chromatin, is the hallmark signature of the S phase of the cell cycle. Other

molecular signatures of the S phase include: 1) the continued maintenance of pRb hyperphosphorylation, 2) the appearance of PCNA proteins at the focal points of DNA replication which act as co-activators of DNA polymerase δ [69], and 3) the increased expression and enzymatic activity of the cdk2-cyclin A heterodimer which acts to cease DNA replication towards the end of the S phase by phosphorylating and inactivating proteins such as E2F1 [70].

3) G_2 and M phases: As mentioned previously, the synthesis of proteins needed for chromosomal segregation during mitosis (i.e., microtubules and microtubule-associated motor proteins) occurs in the G_2 phase [44]. Further, the completion of DNA synthesis and repair are completed prior to the termination of the G_2 phase [44]. A prominent molecular marker which signify that cells are existent in the G_2 phase includes the increased expression and enzymatic activity of the cdk1-cyclin A/B complexes which, as evidenced through knock-out and mutational knock-in cdk1 studies, are obligatory for entry into mitosis [71]. Another manner to detect the G_2 and M phases includes using proliferating cell culture models to detect DNA content with a fluorescent dye or BrdU labeling, and allocating fluorescent-activated cell sorting (FACS) analysis with bioinformatic software modeling to determine which portion of the cells exist in these two phases. While these methodologies have recently been performed in myoblasts that were treated with pharmaceutical agents [72], using FACS-based methods are relatively cost-inefficient and require extraneous amounts of tissue for analyses.

Satellite cell differentiation. Differentiation describes the process of a precursor cell becoming a more specialized cell type. For instance, a proliferating myoblast is a cellular precursor to a mononucleated myocyte (muscle cell), with the latter cell

containing specialized organelles including myofibrils with contractile elements, mitochondria, sarcoplasmic reticulum, and enzymes that are integral for fuel metabolism. If a myoblast does not differentiate following proliferation then it will not possess the functional characteristics of a mature myofiber; this occurrence being due to the fact that undifferentiated myoblasts are obliged to genomically enter differentiation prior to expressing genes that encode the aforementioned functional, muscle-specific proteins.

Physiological cues that induce myoblast differentiation remain relatively obscure compared to the below-mentioned markers of differentiation. In cell culture experiments, removing mitogenic proliferation media (i.e., chick embryo extract- or fetal calf serum-containing medium) and replacing it with DMEM (i.e., a differentiation medium containing amino acids, salts, glucose and vitamins supplemented with insulin as a surrogate growth factor) induces myoblast differentiation [73]. Interpolating these findings to a physiological context suggests that the immediate post-exercise increase in serum and/or muscle-derived growth factors (i.e., MGF, HGF, LIF, etc) acts to induce satellite cell activation and proliferation whereas their eventual disappearance from the muscular milieu may induce a subset of myoblasts to differentiate. Conversely, recent evidence suggests that a delayed (i.e., > 48 hours post-exercise) expression of the IGF-IEa splice variant may act to induce a subset of proliferating myoblasts to differentiate making this gene and its encoded protein extremely relevant markers of interest [21]. In partial support of this hypothesis, Masuro and Rosenthal [74] transfected rat myoblasts with an IGF-1 gene that was under control of the myosin light chain promoter. Using these methodologies, the IGF-1 gene could only be expressed once myoblasts exist in a non-proliferative and genomically differentiated state whereby myosin light chain is able

to be expressed. These authors reported that, compared to non-transfected myoblasts, the post-mitotic expression of IGF-1 induced the expression of myogenin, creatine kinase, and MHC_{emb}; all of these genes being markers of differentiation. Likewise, there is similar mammalian cell culture evidence to suggest that the stimulating the IGF-1 signaling pathway is capable of inducing differentiation [75]. Thus, there is ample evidence to suggest that IGF-1 splice variants can differentially act to increase satellite cell activation, proliferation, and differentiation. Exactly how IGF-IEa signaling mechanistically differs from MGF signaling, however, remains obscure given that both peptides plausibly act through IGF-1 receptor signaling cascades [i.e., MAPK, Akt-mTOR activation, and non-PI-3kinase mechanisms [74]], albeit MGF also appears to induce satellite cell proliferation through an unidentified IGF-1 receptor-dependent pathway [76].

Finally, it has been postulated that the size of myonuclear domains cues myoblast differentiation (depicted in Figure 2). In support of this hypothesis, Petrella et al. [15] state,

> *"…a ceiling size on the [myonuclear] domain has been suggested based upon the concept that sufficient expansion of the myonuclear domain likely puts each nucleus under greater strain to supply the necessary gene products, driving a demand for the addition of new myonuclei to make possible continued growth beyond the domain ceiling."*

Further, these authors have demonstrated that favorable respondents to chronic resistance exercise, or those that exhibited the largest increases in muscle fiber cross-sectional area (CSA) following 16 weeks of conventional resistance (+60%, $p < 0.05$) training, also

exhibited the largest increases in myonuclear addition (+25% in myonuclear number, $p < 0.05$) [15]. It is noteworthy to mention that modest responders (+20% CSA, $p < 0.05$) and non-responders to resistance exercise (no change in CSA, $p > 0.05$) in this same study exhibited a modest increase (+8% in myonuclear number, $p < 0.05$) and no change in myonuclear number, respectively. In attempting to explain these phenomena, the authors inexplicitly stated, *"Expansion of the myonuclear domain may drive myonuclear addition [i.e., differentiation and fusion of myoblasts] by placing strain on existing nuclei to produce adequate gene products for the growing myofiber."* However, these authors have similarly reported that the prevalence of serum/muscle myostatin protein levels and/or the myogenic mRNA expression of *IGF-1EA*, *MGF*, *MYOD*, *MYOGENIN*, *CYCLIN D1*, *MYOSTATIN*, $P27^{KIP1}$, $P21^{CIP1}$, and/or *ACTIVIN IIB* (a component of the myostatin receptor) determine the degree of myofiber hypertrophy, myonuclear number increases, and/or satellite cell activity following prolonged training [14]. Therefore, the identification of an explicit signal(s) originating from post-mitotic nuclei that cues myoblast differentiation/fusion once the myonuclear domain ceiling is breached remains to be determined.

Prominent molecular markers of differentiation include the increased expression and activity of MRFs (of interest MyoD and myogenin), cdk inhibitors (of interest $p21^{Cip1}$ and $p27^{Kip1}$), and pRb [68]. Conversely, there is a decrement in the expression of transcription factors termed inhibitors of DNA binding (i.e., the Id1, Id2, and Id3 isoforms) which bind to and inhibits MRF DNA binding affinity to upstream promoter/enhancer regions; events which abolish MRF-induced increases in muscle-specific gene expression [77]. Of interest, an increase in the expression of $p27^{Kip1}$ and

$p21^{Cip1}$ facilitate differentiation by virtue of binding to and inhibiting the activity of all cdk-cyclin complexes [78]. Likewise, an increase in pRb expression halts the cell cycle by re-sequestering E2F transcription factors which act to induce the expression of proteins needed for DNA synthesis as previously mentioned. Finally, the increased expression and activities of MyoD (during proliferation) and myogenin (estimated to occur 12 hours following the onset of differentiation) occur in proliferating myoblasts that are cued to exit the cell cycle and differentiate [53, 77]. Other molecular markers that indicate early differentiation events include the expression of muscle-specific genes and proteins including creatine kinase [53], desmin [79], alpha-actinin [79], alpha-actin [79], titin [79], troponin-I [79], and MHC_{emb} [31] followed by the expression of adult myosin isoforms [53]. Furthermore, the appearance of organized myofibrils has been reported to occur 15-24 hours following mitosis in proliferating myoblasts that were induced to differentiate [79]. Taking all of the aforementioned findings into consideration (i.e., summating the time needed for proliferation and differentiation to occur) suggests that the *de novo* formation of a differentiated myocyte occurs at least 48 hours following a mitogenic stimulus such as resistance training.

Finally, it is obligatory to describe the multifaceted mechanisms of MRFs due to their purported roles in cuing myoblast differentiation. As mentioned previously, the four MRFs include MyoD, myogenin, Myf5, and MRF4. In short, MRFs are classified as basic helix-loop-helix (bHLH) proteins that bind to ubiquitously expressed E proteins to form transcription factor heterodimers. Once these active heterodimers are formed, they bind to E-box sequences that are present in upstream promoter/enhancer DNA sequences whereby they act in concert with other transcription factors (MEF2C and SRF are

examples) as well as chromatin remodeling enzymes (i.e., SWI-SNF and histone acetyl transferases) to affect the expression of various genes [80]. As alluded to previously, there is a distinct temporal expression of the MRFs during the differentiation process with MyoD and Myf5 being expressed first followed by myogenin and MRF4 [42]. However, transgenic (i.e., ectopic expression) cell culture experiments have indicated that introducing genes that encode each of these MRFs into precursor muscle cells induces differentiation [81]; a phenomenon which suggests that MRFs possess functional redundancy. Likewise, a series of knockout experiments have led scientists to believe that Myf5 is functionally redundant to MyoD and MRF4 is functionally redundant to myogenin [42]. Therefore, it seems as if the expression of MyoD and myogenin are obligatory for differentiation to occur, whereas the expression of Myf5 and MRF4 act functionally superfluous transcription factors for the differentiation process. It has also been hypothesized that the early expression of MyoD acts to "prime" the myoblast for differentiation as well as increase the expression of myogenin, whereas myogenin subsequently acts to induce the expression of muscle-specific structural genes [i.e., genes such as the three myosin heavy chain isoforms which contain E box sequences in their upstream promoter regions [82]] [81]. In regards to MyoD "priming" myoblasts for differentiation, there is ample evidence to suggest that MyoD: 1) induces the expression of pRb [80] and $p21^{Cip1}$ [83], and 2) binds to $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ in such a way as to increase their inhibitory action against cdks [68]. Likewise, there is other evidence to suggest that myogenin is more highly expressed in and regulates the expression of muscle-specific genes in slow oxidative fibers, whereas MyoD assumes this role in fast glycolytic fibers [84]. Thus, all of the aforementioned evidence suggests that is of

particular interest to examine the expression patterns of MyoD and myogenin following an exercise stimulus when interested in detecting the presence of differentiation events.

Satellite cell fusion. The fusion of differentiated myoblasts to pre-existing muscle fibers is perhaps the least understood process of satellite cell physiology. In this regard, Jansen and Pavlath stated in a 2008 review article on the topic that, "*many aspects of mammalian myoblast fusion remain a mystery*" [85]. Rare data in the form of a classical time-lapse TEM- and fluorescent-based microscopic study [86] has delineated various phenotypic patterns that occur in myoblasts prior to fusion with other muscle cells including: 1) a build-up of cytoplasmic vesicles in myoblasts that are in close proximity to the region where fusion occurs, 2) the disappearance of extracellular, membrane-bound proteins and glycoproteins between the sarcolemma interface of the fusing cells, and 3) an internal structural protein breakdown preceding fusion followed by the reorganization and incorporation of these proteins into the complex protein lattice of the mature muscle following fusion. Elucidating explicit myoblast-borne genes and/or paracrine/endocrine signals that regulate these processes have been proven less fruitful, although Jansen and Pavlath provide evidence (i.e., genetic knock-down and antibody interference studies in culture) to suggest that proteins expressed in and present on the sarcolemma of differentiated myoblasts may act to bind to cognate receptors present on post-mitotic fibers and initiate membrane union. These proteins include M-cadherin, NCAM, integrin-beta1, the urokinase plasminogen activator receptor/serine proteinase urokinase plasminogen activator/plasminogen activator inhibitor-1 complex, glucose-related protein 94, caveolin-3, myoferilin, and nicotinic acetylcholine receptors. Nonetheless, several

facets of myoblast-myofiber fusion remain to be determined and, therefore, are beyond the scope of the current investigation.

Other signaling pathways. As a side, it is also important to briefly mention other prominent pathways that are synonymously active with satellite cell activity. The Notch signaling pathway has been suggested to be involved in preventing precocious myoblast differentiation as evidenced in primary human satellite cell cultures, albeit the role of Notch receptors (Notch1, Notch3), Notch ligands (Jagged1, Jagged2, Delta-like1), Notch pathway inhibitors (Numb), and/or Notch-regulated genes (Hes1, Hes6) in this process has not been well established [87]. Likewise, bone morphogenic protein (BMP)/TGF-beta signaling, which transduces intracellular signals through activated SMAD heterodimers, has been shown to inhibit myoblast differentiation [88], and inhibitors of this pathway (specifically the *Twisted gastrulation* and *gremlin* genes) are concomitantly upregulated during myoblast differentiation [77]. Finally, the canonical Wnt signaling pathway operates by the ligand binding of Wnt proteins to the Frizzled family of receptors which, in turn, inhibits the intracellular axin/GSK-3/APC complex that is known to promote the degradation of cytoplasmic beta-catenin proteins. The subsequent accumulation of beta-catenin proteins following Frizzled receptor ligand binding leads to the increased nuclear localization of beta-catenin proteins whereby they act as transcription factors to promote the up-regulation of genes that favor satellite cell proliferation and inhibit myoblast differentiation [89]. Nonetheless, while Notch, BMP/TGF-beta, and Wnt signaling are indeed integral components of satellite cell physiology, elucidating the effects of aging and exercise on these pathways are beyond the scope of the current investigation.

Effects of Exercise on Markers of Satellite Cell Activity

Advanced time-lapse microscopy techniques have enabled multiple investigators to clearly demonstrate that different modes of exercise elicit an increase in satellite cell number (acutely) and post-mitotic myonuclear number (chronically) in mammalian skeletal muscle tissue (summarized in Table 1). Conversely, sparse equivocal evidence does exist suggesting that a strenuous bout of endurance training in rats does not increase MyoD-positively stained cells at 0-72 hours post-exercise leading the authors to conclude that satellite cell activation did not occur at these time points [90]. Contrary to their hypothesis, it is possible that satellite cell proliferation occurred, but none of the proliferating myoblasts expressed MyoD and, therefore, did not commit to differentiation [68]. In this regard, Crameri et al. [65] later demonstrated that an eccentric exercise bout of leg extensions increased NCAM-positive cells (marker of proliferation) whereas there was no increase in the expression of myogenin and/or the neonatal isoform of the myosin heavy chain (markers of differentiation). Thus, this evidence demonstrates the importance of probing explicit biomarkers when attempting to quantitate myoblast proliferation versus differentiation following an exercise stimulus.

Investigators have also quantitated activity-related changes in muscle DNA concentrations without the use of fluorescent microscopy by treating mammalian muscle homogenates with fluorometric Hoechst 33258 dye. For instance, Adams and Haddad [60] have allocated the aforementioned Hoechst dye method to demonstrate that 3 days of functionally overloading the plantaris muscles in rats yielded an approximate 100% increase in DNA without significant increases in muscle protein until day 7. In order to interpret these findings the authors stated, "*Because mature myofiber nuclei are thought*

to be mitotically inactive, increased DNA content in skeletal muscle cells [in this study] suggests activation of satellite cells." Further, our laboratory (*unpublished observations which will be submitted for publication*) has recently allocated the same Hoechst methodologies to demonstrate that an acute bout of conventional resistance training (i.e., 9 sets of lower body exercise training using a lifting intensity of 80% 1RM) with or without pre-exercise nutrient ingestion in college-aged males: 1) increases muscle DNA content by 40% ($p < 0.05$), 2) increases the mRNA expression of *CDK4* by 60% ($p < 0.05$), and 3) decreases the mRNA expression of *MYOD* by 80% ($p < 0.05$) and $P27^{KIP1}$ by 640% ($p < 0.001$); all of these observations occurring 6 hours following exercise. Similar to the contentions of Adams and Haddad [60], our findings provide rare evidence (in particular our tandem DNA and cell cycle regulator gene expression analyses) to suggest that satellite cell activation and proliferation may be occurring at early post-exercise time points following a conventional resistance exercise bout prior to differentiation events (depicted in Figure 4). Therefore, previously published research has reported an increase in the DNA concentrations of muscle homogenates in tandem with other satellite cell markers following mechanical loading; with these findings also suggesting that satellite cell activity increases with mechanical loading.

It is well established that a single bout of resistance exercise elicits an increase in the mRNA and/or protein expression of biomarkers indicative of satellite cell proliferation and differentiation. For instance, a time-course study conducted by Psilander et al [57] demonstrated that *MYOGENIN*, *MYOD*, and *MRF4* mRNA levels were elevated in human skeletal muscle by 100-400% at 0, 1, 2, 6, and 24 hours following a leg press protocol consisting of 4 sets of 6-12 repetitions. Similar findings

have been reported following an acute bout of resistance exercise including: 1) a 4-hour post-exercise increase in *MYOD* mRNA (2.0-fold) and *MRF4* mRNA (1.4-fold) [91], 2) a 12-24-hour post-exercise increase in *MYOD* mRNA (83%), *MYOGENIN* mRNA (3-fold), *CYCLIN D1* mRNA (50%), and $P21^{CIP1}$ mRNA (16-fold) [29], 3) a post-exercise increase of *MRF4* mRNA (3.7- to 4.5-fold, 2–4 hours post), *MYOD* mRNA (5.8-fold, 8 hours post), and *MYOGENIN* mRNA (2.6- and 3.5-fold, 8–12 hours post) [92], 4) a 24-hour post-exercise decrease in $p27^{Kip1}$ (–16% mRNA, –20% protein) and increase cyclin D1 mRNA (34%) [17], 5) a post-exercise increase *MYOD* mRNA (27% and 47%, 0 and 6 hours post), *MYOGENIN* mRNA (24% and 46%, 0 and 6 hours post), MyoD protein (58% and 3.2-fold, 0 and 6 hours post), and myogenin protein (52% and 2.5-fold, 0 and 6 hours post) [93]. Nonetheless, caution should be exercised when interpreting the aforementioned acute data due to the fact that a single bout of resistance exercise has been shown to only increase satellite cell proliferation 1, 4, and 8 days post-exercise (i.e., no study has detected myoblast differentiation/fusion occurring in acute settings) [16, 65]. Taking this data into consideration, exercise-induced increases in MRF mRNA and/or protein expression following a single bout of exercise does not necessarily confer the formation of a differentiated myoblast.

Limited evidence has studied the effects of sequential exercise on the mRNA and protein expression of satellite cell activity markers. Costa et al. [94] demonstrated that six consecutive days of 90 eccentric leg extensor contractions in young males was shown to elicit: 1) a significant decrement in *MYOD* mRNA expression after two bouts and a return to basal levels after 6 bouts, 2) a significant increase in *MYOGENIN* mRNA after two bouts and a return to basal levels after 6 bouts, 3) elicit a significant increase in

P21^{CIP1} mRNA after two bouts and a return to basal levels after 6 bouts, and 4) elicit a significant increase in *KI-67* mRNA (a marker of satellite cell proliferation) after two bouts and a further increase after in this gene 6 bouts. Interestingly, the authors commented upon these findings being peculiar given that the expression of differentiation markers peaked prior to the zenith of *KI-67* mRNA expression. Prior work by Haddad and Adams [95] similarly demonstrated that two sequential exercise bouts (i.e., an electrical stimulation protocol in rats) elicited greater responses in the mRNA expression of cellular proliferation (i.e., *CYCLIN D1*) and differentiation (i.e., *MYOGENIN*) indices at 15-40 hours post-exercise compared to a single exercise bout. Further, these authors reported that the post-exercise phosphorylation/activation of p70S6 kinase was elevated for up to 40 hours following the second stimulation bout if these animals were allowed 24-48 hours between sequential stimulation sessions, whereas one session alone did not elicit and increase in p70S6 kinase activation. The latter finding is of the upmost relevance due to the fact that p70S6 kinase activates ribosomal protein S6 which, in turn, acts to increase the translation of mRNAs containing 5' terminal oligopyrimidine tract (5' TOP) sequences at the transcriptional start site including ribosomal-protein mRNAs. The end result of this process includes an increase in ribosomal number or translational capacity. In a recent editorial paper, George Thomas provides compelling evidence from bacteria, yeast, and amphibians to suggest that cellular growth must precede proliferation and differentiation due to the fact that a premature proliferative response in cells that have not adequately grown will yield progeny that are not able to differentiate [96]. In this regard, Thomas contends that ribosomal biogenesis (i.e., the translation of ribosomal-protein mRNAs yielding protein synthetic machinery) must occur prior to proliferation

given that protein synthetic machinery confers cellular growth by increasing the translation capacity of growing cells. Thomas argues,

> *"In a mitogen-stimulated cell, ribosome biogenesis is initially selectively upregulated, through the recruitment of ribosomal-protein mRNAs onto polysomes. However, as more ribosomes are generated and the translational capacity increases, the pathway becomes desensitized favouring the translation of other mRNAs. Thus, ribosomal-protein mRNAs first serve to increase protein synthesis capacity and the prevent the translation of mRNAs lacking a 5' TOP, such as cell cycle regulators, until an appropriate pool of ribosomes have accumulated."*

Finally, Dr. Esser's group recently demonstrated that mTORC1 activation via the stimulation of differentiated myotubes with IGF-1 resulted in the concomitant elevation of cyclin D1 protein and rRNA concentrations [97]. In an attempt to integrate all of their findings, these authors explained that IGF-1 activates mTOR which: 1) increases the translation of cyclin D1 mRNA which acts in concert with cdk4 to increase the expression of rRNA by activating transcriptional activators upstream of rDNA genes, and 2) increases the activation of p70S6 kinase and translation of ribosomal protein mRNAs. Thus, it becomes plausible that the IGF-1-mediated activation of p70S6 kinase, ribosomal protein S6 activation, and an "adequate" amount of ribosomal biogenesis may require multiple bouts of exercise to occur in satellite cells; an effect which may delay the translation of differentiation-regulating transcripts (i.e., MRF mRNAs) and the process of myogenesis itself. Nonetheless, the aforementioned evidence again suggests that: 1) multiple bouts of exercise may compound the increase the expression of satellite cell

activity regulators in comparison to one exercise bout, 2) an increase in the satellite cell polyribosome population following one or multiple exercise bouts may be needed prior to MRF mRNAs being translated, and 3) the exercise-induced increases in MRF mRNA expression following a one or multiple bouts of exercise do not indicate the *de novo* formation and fusion of differentiated myoblasts to pre-existing myofibers. Thus, it is imperative that other biomarkers of translational capacity (i.e., total RNA), proliferation (i.e., immunohistochemistry and/or DNA quantitation) and differentiation (i.e., immunohistochemistry, [DNA]: [protein] quantitation, and/or quantitating proteins indicative of differentiation) need to be assessed in tandem with the expression of cell cycle/differentiation-regulating transcripts to further delineate if these processes have occurred following exercise.

It is relevant to comment upon whether or not the exercise-induced expression of various cell cycle and differentiation regulators occurs from satellite cells alone versus post-mitotic fibers and satellite cells (as indicated in Figure 3). The percent of myonuclei contributed from satellite cells to the total nuclei pool in skeletal muscle samples has been reported to be minimal. In this regard, Karyn Esser and her research group [98] have contended that the post-exercise expression of myogenic (i.e., cyclins and MRFs) and anti-growth (i.e., cdk-inhibitors) mRNA transcripts likely originate from post-mitotic fibers due to the fact that satellite cell nuclei account for merely 1.8% of the total nuclei in the muscle bed compared to post-mitotic fiber nuclei which constitutes ~43% of the total nuclei pool. To support their contention, the authors stated, "*Therefore, if the cell cycle gene expression changes come from the subset of satellite cells that are activated following contraction then those cells must be expressing those genes very highly for*

them to be detectable in a homogenate from whole [muscle]." Alternatively, these authors postulate that the expression of anti-growth genes (i.e., cdk-inhibitors, MRFs) occurs in post-mitotic myonuclei in order to "counter" the cellular proliferation genes (i.e., cyclins) being expressed from these same nuclei. Further, these authors contend that the post-exercise increase in MRF expression from post-mitotic nuclei may occur to keep muscle cells in a differentiated, non-proliferative state. In support of this hypothesis, Esser's group has provided evidence to suggest that differentiated myotubes express *CYCLIN D1* in culture when treated with mitogenic culture media [97]. To counter this argument, however, there is recent cell culture data to suggest that the expression of cyclins minimally occurs in terminally-differentiated fibers maintained in differentiation media [99]. Likewise, a recent study allocated *in situ* RNA hybridization techniques, which uses microscopic methods to localize mRNA transcripts in different cellular regions, to demonstrate that none of the MRFs are expressed in the mature myofibers of *mdx* mice and are, instead, highly expressed in proliferating satellite cells [100]. Taking all of this data into consideration it seems as if the mRNA and protein expression of genes that control cellular proliferation and differentiation are expressed and localized in satellite cells, respectively, albeit the aforementioned equivocal evidence deems it obligatory to perform future research using *in situ* RNA hybridization techniques or FACS-based methods following an exercise stimulus to unequivocally delineate which human cell type expresses the aforementioned markers.

Effects of Aging on the Expression of MRFs and Markers of Satellite Cell Activity at Rest and following Resistance Exercise

Several histological and molecular manifestations accompany muscle aging. A classical cadaver study performed by Dr. Jan Lexell demonstrated that vastus lateralis fiber number and muscle size are diminished in older versus younger male humans (364,000 versus 478,000 fibers, $p < 0.01$; gross muscle size was18% smaller, $p < 0.01$), providing strong evidence that muscle fibers unexplainably disappear with aging. Likewise, Haddad and Adams [22] have determined that rodent muscle, while containing similar total protein content, possess a significantly lower amount of myofibrillar protein content when compared to younger counterparts. Finally, satellite cell numbers are purportedly reduced in older humans [specifically, Verdijk et al. [3] reported that type II fiber satellite cells are 45% lower in older humans] and rodents [101, 102]. In regards to the latter findings, *in vivo* evidence suggests that satellite cells undergo 1-2 divisions prior to committing to differentiation [103]; this being a phenomenon which advocates the notion that satellite cell pools may become mitotically exhausted over a lifetime. However, the evidence presented below also suggests that age-related decrements in satellite cell number and proliferative potential are likely caused by other molecular mechanisms as well.

Although the mechanisms leading to decrements in myofibrillar protein and myofiber number are poorly understood, it has been hypothesized that decrements in satellite cell activity with aging [i.e., proliferation [104] and differentiation [19, 105]] may serve as the causal hub of these events. In this regard, Gopinath and Rando [106] authored an extensive review which provides evidence to suggest that the negative

alterations in circulatory factors (i.e., leukocytes as well as known and unknown hormones and growth factors) as well as the extracellular matrix surrounding satellite cells occurs with aging. In support of the former contention, Rando's research group conducted an experiment using heterochronic parabiosis whereby pairs of young and old mice were surgically "combined" to share a common circulatory system [107]. Interestingly, performing this procedure over a 5-week period significantly enhanced the proliferative capacity of satellite cells of older mice 24 hours following muscle injury in culture. When explaining these findings, the authors stated,

> *"Our experiments suggest that there are systemic factors that can modulate the molecular signaling pathways critical to the activation of tissue-specific progenitor cells, and that the systemic environment of a young animal is one that promotes successful regeneration, whereas that of an older animal either fails to promote or actively inhibits successful tissue regeneration."*

Contrary to the aforementioned hypothesis, recent evidence also suggests that co-culturing satellite cells from young rats on tissue dishes coated with the extracellular matrix extract from old rats actually enhances proliferation capabilities [108]. These authors deemed as these findings as surprising and postulated that they may be due to the lower efficiency at which extracellular matrix components can be isolated from older rats; this being a methodological limitation which may have obscured their findings. Nonetheless, there is equivocal evidence to suggest that mitotically senescent fibroblasts present in the extracellular matrix of skeletal muscle in older species secrete elevated levels of proinflammatory cytokines (i.e., TNF-alpha and IL-1beta are examples) which could impair satellite cell proliferation and/or differentiation [109, 110].

Contrary to the abovementioned age-associated alterations in circulating and extracellular matrix factors, there is convincing evidence to also suggest that the age-associated decrement in the expression of muscle-derived IGF-1 variants is causally linked to satellite cell dysfunction due to its integral role in satellite cell activation. In this regard, Machida and Booth [111] authored a review article on the topic stating, *"…satellite cells in skeletal muscle of [old] rats are in sufficient quantity, but inactive as a result of lack of some endogenous growth factors, possibly including IGF-1."* Data from Booth's laboratory demonstrating that IGF-1 administration to aged rats substantially increased satellite cell proliferation supports this hypothesis [112]. Furthermore, past literature has determined that older rodents and humans exhibit a decrement in *MGF* and/or *IGF-1EA* mRNA expression following an acute bout of exercise [10, 15, 22], and limited human evidence [15, 23] has suggested that the acute post-exercise decrement in *IGF-1* mRNA expression is associated with a long-term decrement in phenotypic adaptations (i.e., increases in muscle CSA) accrued from resistance training. Finally, our laboratory has recently determined that the baseline myogenic expression of *IGF-IEA* mRNA is significantly lower in older versus younger humans during resting states [113]. Taking all of this evidence in consideration, it would be insightful to determine if a potential age-related decrement in basal and post-exercise MGF and IGF-1Ea mRNA/protein expression is associated with a lag in cell cycle regulators and markers of *de novo* satellite cell replication during a series of resistance training sessions.

In regards to skeletal muscle aging, limited evidence suggests that MRF mRNA (i.e., *MYF5* ↑82%, *MYOD* ↑58%, *MYOGENIN* ↑41%, *MRF4* ↑82%) [91] and protein (i.e.,

myogenin ↑44%) [18] are generally higher in the skeletal muscle of older individuals; an effect which may be due to aging skeletal muscle attempting ameliorate muscle loss through the compensatory expression of these myogenic genes [18]. Conversely, intramuscular protein concentrations of cell cycle regulators (i.e., cyclin D1, cyclin B1, and $p27^{Kip1}$) have not been shown to be influenced by human aging [18], albeit a recent investigation by Lees et al [19] reported a 50% decrement in $p27^{Kip1}$ protein levels from older cultured rat satellite cells. Likewise, a similar study demonstrated that a decrement in the $P57^{KIP2}$ and $P21^{CIP1}$ genes occurs in the skeletal muscle of mice during aging; an effect which may contribute to a diminished capability of satellite cells to differentiate [30]. Interestingly, only one study to date [17] has examined the effects of resistance training on the mRNA expression of selected cyclins, cdks, cdk-inhibitors, and MRFs between young and older humans 24 hours following a single bout of conventional resistance exercise. These authors reported that the mRNA expression patterns significantly decreased for $P27^{KIP1}$ (-16%) and increased for *CYCLIN D1* (34%) and *MGF* (49%) in both age groups. However, a *post hoc* analysis revealed a significant increase in *MGF* after exercise in young men only (91%). Hence, it is apparent that there are: 1) loading-mediated effects on MGF, cdk, cyclins, cdk-inhibitor and MRF expression patterns following exercise, 2) "older" satellite cells may express less cdk-inhibitors at and more MRFs baseline, and 3) older skeletal muscle tissue may express less *MGF* following mechanical loading. In this regard, data from the current investigation provides rare evidence of these potential patterns 24-48 hours following three sequential bouts of conventional resistance training.

Concluding Remarks and Significance of the Current Study

Based upon the aforementioned literature, it is apparent that exercise is capable of increasing the number of resident satellite cells in the muscle bed of younger and older individuals, albeit many of these investigations have employed non-conventional and impractical means of mechanical loading (i.e., eccentric exercise). Furthermore, while the various molecular mechanisms that govern the cell cycle have been elucidated, evidence is lacking in regards to the molecular mechanisms that decrease satellite cell replication capabilities in older versus younger individuals following conventional resistance exercise. The transient post-exercise myogenic expression of *MGF* has been shown to be impaired in older versus humans younger [10, 17], albeit these studies have not examined the plausible downstream manifestations that may occur in response to this age-divergent mechanism. In this regard, the current investigation determined *MGF/IGF-1EA*, *CYCLIN D1*, cdk, cdk-inhibitor, and *MRF/IGF-1EA* mRNA expression patterns as well as the appearance of markers signifying the *de novo* synthesis of satellite cells prior to and following one week of conventional resistance exercise. As mentioned previously, the findings from this study warrants future research in determining the potential epigenetic modifications that may occur in aging satellite cells and/or post mitotic muscle fibers.

CHAPTER III

METHODS

Participants

Based on the findings of Kim et al. [17], which determined the 24-hour post-exercise changes in *MGF* mRNA expression to be significantly altered between ages (young = 1.03 ± 0.11 AUs, old = 0.86 ± 0.11 AUs), the sample size calculations for a between-subjects study design yielded a minimum sample size of n = 7 for each group in order to attain a statistical power of 0.80. Therefore, to minimize the probability of making a type II error, 10 older (60-75 y) and 10 younger (18-25 y) males who were not currently participating in any form of resistance training with their lower bodies were recruited for this study. Eligibility inclusion criteria included the following: 1) participants were apparently healthy but cannot have participated in a structured lower-body resistance exercise regimen consistently (i.e., at least one time per week) one year prior to participation in this study, 2) participants were instructed to abstain from smoking, alcohol, tobacco or caffeine for the duration of the study, 3) participants cannot have consumed ergogenic nutritional supplements for at least three months prior to the start of the study, 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 any orthopedic problems (e.g., previous surgery, joint replacements, etc.) or cannot have been previously diagnosed neuromuscular disease that prevented them from participating in the resistance

training sessions, 7) participants could not be taking prescription medications indicated for heart, pulmonary, anti-coagulant, anti-hypertensive, psychotropic, neuromuscular/neurological, or androgenic dysfunctions, 8) participants could 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 submitted the University of Oklahoma Health Sciences Center Institutional Review Boards for Human Subjects for approval and all participants completed a written informed consent form (Appendix D) as well as a pre-study health and exercise status questionnaire (Appendix E). All participants who were the older group were required to provide written medical clearance from their primary care physician using the form provided (Appendix F).

Research Design

An overview of the research design is presented in Figure 5. A two-way [age (young vs old males) x testing session (T1-T4)] repeated measures design was used to determine the effects of age and repeated bouts of conventional lower body resistance training on markers indicative of satellite cell proliferation (i.e., muscle [DNA], [c-met], [PCNA], and the fold-change expression in *CYCLIN D1*, *CDK4*, *CDK2* mRNA transcripts), differentiation (i.e., [myofibrillar protein], [total protein], [total protein]:[DNA], the fold-change expression in MHC_{EMB} and *MYOD* mRNA transcripts), and growth factor expression (i.e., muscle [IGF-1], and the fold-change in *MGF* and *IGF-1EA* mRNA transcripts). All participants visited the laboratory seven times (T1 = day 0, 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 verbally screened to ensure that each person met the entrance criteria. Furthermore, participants completed the informed consent and health and history questionnaire prior to first visit. During the first visit (T1), participants reported to the laboratory between 0600 and 0900 following a 12-hour fast and had a percutaneous muscle biopsy 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 r.p.m. at a self-selected intensity) and performed 1RM strength testing on bilateral leg press, hack squat, leg extension machines using guidelines established by the National Strength and Conditioning Association [114]. For the leg press and hack squat exercises, a successful repetition required that each participant attained a 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 FAM1 whereby they warmed up for 5 minutes of a cycle ergometer and performed 2 sets of 10 repetitions at a lifting intensity of 60% 1RM for each of the three lower-body exercises with 2-3 minutes alotted between sets. Two days following FAM1, participants returned to the laboratory for FAM2 whereby they warmed up for 5 minutes of a cycle ergometer and performed 2 sets of 10 repetitions at a lifting intensity of 70% 1RM for each of the three lower-body exercises with 2-3 minutes alotted between sets. The purposes of FAM1 and FAM2 were to ensure that participants were gradually eased into the training protocol employed during workout 1 through T3, and to minimize the delayed onset of muscle soreness with these untrained participants which has been employed by Bamman and colleagues [18]. Two days following FAM2, participants reported to the laboratory for workout 1 between 0600 and 0900 following a 12-hour fast,

warmed up for 5 minutes on a bicycle ergometer, and performed 3 sets of 10 repetitions at a lifting intensity of 80% 1RM for each of the three lower-body exercises with 2-3 minutes alotted between sets. Two days following workout 1, participants reported to the laboratory for T2 between 0600 and 0900 following a 12-hour fast, donated a second muscle biopsy from the opposite leg of T1, warmed up for 5 minutes of a cycle ergometer, and performed 3 sets of 10 repetitions at a lifting intensity of 80% 1RM for each of the three lower-body exercises with 2-3 minutes alotted between sets. Two days following T2, participants reported to the laboratory for T3 between 0600 and 0900 following a 12-hour fast, donated a third muscle biopsy from the opposite leg of T2, warmed up for 5 minutes of a cycle ergometer, and performed 3 sets of 10 repetitions at a lifting intensity of 80% 1RM for each of the three lower-body exercises with 2-3 minutes alotted between sets. Finally, one day following T3, participants reported the laboratory for T4 between 0600 and 0900 following a 12-hour fast and donated a fourth muscle biopsy from the opposite leg of T3. It should be noted that all participants were verbally encouraged during each set by a laboratory technician. As mentioned previously, all leg press and hack squat repetitions required that each participant attains 90º knee flexion at the bottom (eccentric) portion of the repetiton which was visually gauged by a laboratory technician. Furthermore, it should be noted that participants were able to complete all workouts without detracting weight from their prescribed lifting volumes.

Variables

The independent variable in this study is age group (i.e., younger vs older). The following genes, proteins, and other biomarkers were examined at each muscle biopsy

time point and served as dependent variables. Note that the following descriptions have been adapted from Chapter II of this paper.

General Biomarkers of Satellite Cell Differentiation

1. *Muscle [DNA]*. For T1-T4 for each participant; a post-exercise increase in this marker indicated that DNA synthesis has occurred in the muscle bed; potentially from proliferating myoblasts. Wong and Booth [115] demonstrated that DNA content from the gastrocnemius significantly increased by 20% ($p < 0.05$) 12 and 36 hours following exercise in rats that performed 24 weighted concentric contractions. While these authors attributed these increases to an increase in satellite cell activity, they also stated that infiltrating leukocytes following exercise may have also contributed to increases in DNA content. A follow-up study performed by Malm et al. [116] used immunofluorescent microscopy to determine if eccentric loading increased the infiltration of leukocytes into skeletal muscle at various post-exercise time points. Their findings suggest that satellite cell activation (i.e., CD56+ cells) increased 271% ($p < 0.05$) 48 hours following exercise. Likewise, macrophage infiltration (CD163+ cells) also increased and was the predominant signal of post-exercise leukocyte infiltration in the muscle bed at this time point. Therefore, protein homogenates from each biopsy were also probed for CD163 to confirm that post-exercise elevations in DNA content were not predominantly due to infiltrating monocytes/macrophages.
2. *Muscle [myofibrillar protein]*. For T1-T4 for each participant; the myofibrillar protein pool includes sarcomeric proteins such as the various isoforms of myosin, actin, troponin, nebulin, titin, etc. Increases in myofibrillar protein may partially signify increases in myofibrillar protein synthesis in differentiating fibers. The

predominant limitation of quantitating this pool using our methods is the non-discriminatory detection of newly synthesized myofibrillar protein that may arise from pre-existing fibers. In this regard, other genetic and proteomic markers (i.e., fold-changes in MyoD and MHC_{emb} mRNA transcript expression) were analyzed to further delineate if myoblast differentiation/fusion potentially occurred.

3. *Muscle [total protein]*. For T1-T4 for each participant; the total muscle protein pool includes myofibrillar, sarcoplasmic, mitochondrial, and nuclear sub-fractions as well as extracellular matrix proteins. Again, increases in this biomarker following exercise may have signified an increase in satellite cell differentiation and fusion events although the aforementioned limitations still exist.

Muscle mRNA Transcripts

In a recent review article [117] geneticist Dr. Frank Booth states, "*As a general rule, changes in [mRNA] and [protein] encoded by these mRNAs occur in the same direction.*" Therefore, it is my contention that detecting an alteration in the post-exercise mRNA expression of cell cycle regulators in concert with other molecular markers (i.e., skeletal muscle [DNA], [c-met], and [PCNA]) provided invaluable insight in regards to which one of the below-mentioned targets were dysregulated with aging.

1. *MGF*. For T1-T4 for each participant; this gene contains a 52 base pair insert relative to the systemic *IGF-1EA* gene and, thus, encodes the *IGF-1EC/MGF* muscle-specific peptide that has an altered C-terminus compared to the IGF-1Ea peptide. The MGF peptide has been shown to operate through IGF-1 receptor-independent pathway to induce satellite cell proliferation, and its mRNA expression is thought to occur transiently (i.e., within 24 hours) following mechanical loading.

2. *IGF-1EA*. For T1-T4 for each participant; this mRNA encodes the systemic IGF-1 peptide which is thought to be synthesized and released from exercised muscle in a delayed fashion following exercise in order to induce a subset of proliferating myoblasts to differentiate [21].
3. *CDK4*. For T1-T4 for each participant; this mRNA encodes the catalytic portion of cdk4-cyclin D1 heterodimer that hyperphosphorylates pRb and facilitates satellite cell entry into the S phase.
4. *CDK2*. For T1-T4 for each participant; this mRNA encodes the catalytic portion of cdk2-cyclin E heterodimer that irreversibly hyperphosphorylates pRb and allows the replicating satellite cell to finish the S phase.
5. *CYCLIN D1*. For T1-T4 for each participant; this mRNA encodes the regulatory portion of cdk4/6-cyclin D1 complexes that hyperphosphorylate pRb proteins allowing the cell to enter the S phase. Furthermore, IGF-1 has been shown to stimulate the mRNA and protein expression of this cell cycle regulator [27, 97].
6. *MYOD*. For T1-T4 for each participant; this mRNA encodes the MyoD myogenic regulatory transcription factor which is expressed in satellite cells that are committed to differentiation.
7. MHC_{EMB}. For T1-T4 for each participant; this myosin isoform has been shown to be expressed in very small regenerating fibers and is thought to signify the presence of newly differentiated myotubes that fuse to pre-existing muscle fibers [31].
8. $P21^{CIP1}$. For T1-T4 for each participant; this mRNA encodes the "shotgun" $p21^{Cip1}$ cdk-inhibitor which binds to cdk-cyclin heterodimers to inhibit their activity; an effect which facilitates cell cycle exit and is needed for differentiation to occur; there

is evidence to also suggest that $p21^{Cip1}$ may actually confer the stability of G1-phase cyclins and, thus, facilitate the cell cycle.

9. *$P27^{KIP1}$*. For T1-T4 for each participant; this mRNA encodes the "shotgun" $p27^{Kip1}$ cdk-inhibitor which binds to cdk-cyclin heterodimers to inhibit their activity; an effect which facilitates cell cycle exit and is needed for differentiation to occur.
10. *Beta-2 microglobulin 9(B2M) and 28s rRNA (28S)*. For T1-T4 for each participant; these mRNAs are stably expressed prior to and following exercise as well as across aging males which makes it a great candidate as a normalizing control when performing gene expression experiments.

Muscle Proteins

1. *Muscle [c-met]*. For T1-T4 for each participant; this protein is explicitly expressed in mitotically quiescent satellite cells and proliferating myoblasts [55]. Observing a concomitant increase in this protein with increases in DNA suggested that satellite cell content is likely increasing following the exercise intervention.
2. *Muscle [PCNA]*. For T1-T4 for each participant; this protein is explicitly expressed in mitotically active satellite cells and signifies the presence of myoblasts undergoing DNA synthesis.
3. *Muscle [IGF-1]*. For T1-T4 for each participant; this growth factor is the resultant peptide of *MGF* and *IGF-1EA* translation (i.e., manufactured antibodies cannot distinguish between the MGF and IGF-IEa isotypes). Nonetheless, an increase in the concentration of this growth factor in skeletal muscle following a series of exercise bouts would suggest that the increased translation of MGF and/or IGF-IEa is occurring.

Instrumentation

1. *Leg press/hack squat combo (Model #: HLS – 160, Yukon Fitness Equipment, Cleveland, OH)*. This machine was used to 1RM test participants for the leg press and hack squat exercises. Further, this machine was used to train participants during subsequent workouts for these respective exercises.
2. *Leg extension machine (Model #: Nautilus NT 1220 Rotary Leg Extension/Curl Station, Nautilus Inc., Vancouver, WA).* This machine was used to 1RM test participants for the leg extension exercise. Further, this machine was used to train participants during subsequent workouts for this respective exercise.
3. *Fluorometer (Model #: Versafluor Fluorometer, Bio-Rad Laboratories, Hercules, CA)*. This machine was used to quantitate DNA from crude muscle homogenates with Hoechst 33258 dye (Sigma, St Louis, MO) being used as the probe.
4. *Automated electrophoresis platform (Model #: Experion Electrophoresis Station, BioRad Laboratories, Hercules, CA)*. This machine was used to randomly test RNA quality of a subset of samples from the aqueous fraction of the Tri reagent (Sigma, St. Louis, MO) homogenates.
5. *96 well spectrophotometer (Model #: Model 680, Bio-Rad Laboratories)*. This machine was used to quantitate total protein and myofibrillar protein from crude muscle homogenates with Bradford dye (Bio-Rad Laboratories) being used as the probe.
6. *Thermal cycler (Model #: MyiQ Optics Model, Bio-Rad Laboratories)*. This machine was used to quantitate baseline and fold-changes in the mRNA expression of the

GOIs from reverse transcribed mRNA present in the total RNA pool (a.k.a., complimentary DNA or cDNA) with SYBR green (Bio-Rad Laboratories) and gene-specific primers (Integrated DNA Technologies, Coralville, IA) being used as probes.

7. *Electrophoresis cell (Model #: Criterion Cell, Bio-Rad Laboratories)*. This machine was used to separate proteins from the cell lysis homogenates based upon molecular mass. Following this step, muscle-specific proteins (i.e., c-met, PCNA, IGF-1) were electrotransferred onto nitrocellulose membranes, probed for transfer efficiency using Ponceau S stain (Sigma, St. Louis, MO), and these proteins were immunoprobed using protein-specific antibodies (c-met, PCNA: Cell Signaling Technology Inc., Manvers, MA; IGF-1: Abcam, Cambridge, MA) and enhanced chemiluminescent reagent (Bio-Rad Laboratories)
8. *Electrotransfer cell (Model #: Criterion Blotter, Bio-Rad Laboratories)*. This machine was used to transfer proteins from SDS-PAGE gels to nitrocellulose membranes as mentioned above.
9. *Gel documentation system (Model #: Chemi Doc XRS, Bio-Rad)*. This machine was used to detect and quantitate protein banding to determine muscle-specific protein expression patterns.

Percutaneous Muscle Biopsies

Biopsies were obtained during T1 (baseline), prior to exercise during T2 (48 hours following workout 1) and T3 (48 hours following workout 2), and during T4 (24 hours following workout 3). 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 [116], biopsies were collected from alternating legs during subsequent testing sessions in such a way that two muscle collections occurred from each leg. It should be noted that previous research examining an exercised versus non-exercised leg in the same participants has revealed that 9 weeks of unilateral leg extensor resistance training has no effect on satellite cell activity in the non-exercised leg [118]. 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 clean of leg 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 a suction apparatus applied to its end was inserted into the pilot hole. Suction was applied and the muscle tissue was excised in a double-chop fasion. Immediately following the biopsy, the muscle tissue was removed from the needle using sterile instruments, the collected tissue was placed into a blindly-labeled cryogenic storage tube, and the tube was dropped in storage container containing liquid nitrogen (i.e., flash frozen). Samples were then transferred for long-term storage into a -80 C freezer until completion of the study for biochemical analyses.

Muscle [DNA] Determination

Using the fluorescent Hoechst dye, increases in muscle [DNA] due to increases in DNA synthesis from satellite cell activation and myoblast proliferation can be inferred.

Briefly, a section of muscle from each time point per participant was weighed (~20 mg) and homogenized on ice with 400-700 µl of cell lysis buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, 10 mM Tris-HCl, pH 6.8) using a tight-fitting pestle. For muscle DNA determination, 50 µl of cell lysis crude homogenate was assayed with 1 ml of a fluorometric dye (Hoechst 33258 dye; Sigma, St Louis, MO), and the fluorescent signal was detected using a single cuvette-based fluorometer (Bio-Rad Laboratories). The buffer for this assay contains a high salt concentration (2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4), and under the high salt condition the dye binds only to the DNA and not the RNA component in the homogenate. Further, this dye is reported to have a high affinity for AT-rich sequences in the minor groove of double-stranded DNA and, when excited at 360 nm, emits a fluorescent signal that is detectable with a 460 nm filter. This method has been successfully employed by Adams and Haddad [60] to detect changes in DNA content within the skeletal muscle of rats following a mechanical loading protocol. The predominant limitation to this method, as proposed by Booth [119], includes quantifying DNA from other cells in the muscle bed including stromal vascular cell lines (i.e., fibroblasts, smooth muscle, epithelial cells) infiltrating leukocytes, intramuscular fat pads as well as DNA from pre-existing muscle. To minimize this interference, excess adipose tissue, connective tissue, and blood cell infiltrates were removed from the muscle samples dedicated to this analysis using sterile instruments. Similarly, the presence of protein markers specific to infiltrating macrophages (i.e., CD163) using dot-blotting techniques were performed to examine if repeated bouts of exercise increase the accumulation of these cells in the muscle bed. Unknown [DNA] values were compared to known [DNA] values using a standard curve method with calf thymus DNA, and all

assays were performed in duplicate. Coefficient of variation values between duplicates averaged 3.3%.

Muscle [Total Protein] and [Myofibrillar Protein] Determination

For muscle [total protein] determination, 50 µl of the aforementioned cell lysis crude homogenate that was sampled for DNA was diluted with 350 µl of dH_2O and 10 µl of this total protein diluent was assayed with 200 µl Bradford reagent (Bio-Rad Laboratories). This method has been successfully employed by Haddad and Adams [22] to detect age-related differences of [total protein] within the skeletal muscle of rats. Absorbance values for each sample were read using a 595 nm filter in a 96 well spectrophotometer (Bio-Rad Laboratories), unknown [total protein] values were compared to known [total protein] values using a standard curve method with bovine serum albumin, and all assays were performed in duplicate. Coefficient of variation CT values between duplicates averaged to be less than 10%.

For muscle [myofibrillar protein] determination, the remainder of the aforementioned crude homogenate that was sampled for [DNA] and [total protein] was centrifuged at 5,000 revolutions per minute (RPM) at 4 C for 10 minutes. The resultant supernatant was discarded and the resultant pellet was treated with 1 ml of myofibril protein extraction buffer 2 (175 mM KCl, 2 mM EDTA, 0.5% Triton-X 100, 10 mM Tris HCl, pH 6.8) vortexed rigorously, and centrifuged at 5,000 RPM at 4 C for 10 minutes. The resultant supernatant was again discarded and the resultant pellet was treated with 1 ml of myofibril protein extraction buffer 3 (150 mM KCl, 10 mM Tris HCl, pH 7.0) vortexed rigorously, and centrifuged at 5,000 RPM at 4 C for 10 minutes. The resultant

supernatant was again discarded and the resultant pellet (made up of pure myofibrils) was suspended in 500 µl of myofibril protein extraction buffer 3 (150 mM KCl, 10 mM Tris HCl, pH 7.0). Fifty (50) µl of the suspended myofibril solution from each sample was assayed using 1 ml of Bradford reagent (Bio-Rad Laboratories), and absorbance values for each sample was read using a 595 nm filter in a single cuvette spectrophotometer (Bio-Rad Laboratories). This method has been successfully employed by Haddad and Adams [22] to detect age-related differences of [myofibrillar protein] within the skeletal muscle of rats. Unknown [myofibrillar protein] values were compared to known [protein] values using a standard curve method with bovine serum albumin, and all assays were performed in duplicate. Coefficient of variation CT values between duplicates averaged to be less than 10%.

Muscle [Total RNA] Determination

Briefly, a second section of muscle from each time point per participant was weighed (~30 mg) and homogenized using 500 µl of Tri reagent (Sigma Chemical Co., St. Louis, MO) which contains a monophasic solution of phenol and guanidine isothiocyanate. Following thorough homogenization, samples were centrifuged at 12,000 RPM 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, samples were vortexed for 15 seconds, and 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 RPM at 4 C for 15 minutes to form a RNA pellet and the resulting supernatant was disposed. The RNA pellet was then exposed to a subsequent 750 µl 75% ethanol wash, centrifuged at 12,000 RPM at 4 C for 5 minutes, and the resulting supernatant was disposed. Finally, the resultant air-dried RNA pellet was dissolved in 50 µl of RNase-free water with repeated pipetting/vortexing. The diluted RNA samples were then stored at -80 C until later analyses.

An aliquot of total RNA per sample was diluted 25-fold and concentrations as well as RNA purity for each sample determined using a spectrophotometer (Bio-Rad Laboratories). The average coefficient of variation between duplicates was 9.0%. A random subset of RNA samples were tested for RNA quality using the proprietary High Sensitivity RNA analysis kit with the Experion Automated Electrophoresis platform (Bio-Rad Laboratories) (Figure 6). 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. 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. All of these aforementioned assays were performed in duplicate.

Real-Time PCR to Detect Pre- and Post-Exercise Expression of Genes of Interest

Following total RNA concentration determination, 50 ng of total RNA was reverse transcribed to synthesize cDNA. For each sample, a reverse transcription reaction mixture [40 μl total: 1) 50 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)] 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 the resultant volume 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) and synthesized through a commercial manufacturer (Integrated DNA Technologies, Coralville, IA) (Table 2). The arithmetic mean of *B2M and 28S* (*B2M & 28S* avg) mRNA was used as an internal reference for detecting the relative quantity of target mRNA due to the fact that our laboratory and others [120] have found these genes to be constitutively expressed after resistance exercise and during muscle aging. Two (2) μl of cDNA were added to each of the separate PCR reactions for *CDK4*, *CDK2*, *CYCLIN D1*, *MYOD*, *MYOGENIN*, $P21^{CIP1}$, $P27^{KIP1}$, *IGF-1EA*, *MGF*, MHC_{EMB}, *B2M* , and *28S* and each PCR reaction contained the following mixtures: 12.5 μl of SYBR Green Supermix (Bio-Rad Laboratories) (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_2O. 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 & 28S). Coefficient of variation for values between duplicates were less than 5% for all GOI's and HKG's.

Protein immunoblotting

Briefly, a third section of muscle from each time point per 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 a protease inhibitor cocktail (Sigma, St. Louis, MO)]. Prior to SDS-PAGE, protein concentrations of cell lysis homogenates were determined spectrophotometrically using Bradford reagent (Bio-Rad Laboratories) with BSA being used as a standard curve. Following protein concentration determinations for each sample, a total of 30 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 minigels (Bio-Rad Laboratories) for SDS-PAGE. Each run lasted 75 minutes at 150 V until the bromophenol blue tracer dye from the Laemmli buffer migrated ~1 cm to the bottom of the gel. Following SDS-PAGE runs, proteins were

eluted from the gels onto nitrocellulose membranes using a "wet transfer" in an electrotransfer blotting apparatus (Bio-Rad Laboratories) with Towbin electrotransfer buffer. Electrotransfers lasted 1 hour in duration at 100 V/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 (TBST), and stored at 4 C overnight in TBST spiked with 5% nonfat blocking agent (NFBA) until immunoprobing experiments were performed. The lack of consistent muscle [c-met] and [IGF-1] signals during pilot Western blotting experiments obviated the need to: 1) immunoprobe c-met using slot-blotting methods whereby protein aliquots were loaded directly onto nitrocellulose membranes and probed as described below, and 2) detect muscle [IGF-1] using commercial enzyme immunoassay (EIA) methods. For muscle [c-met] it should be noted that the secondary antibody solution was confirmed to yield no chemiluminescent signal and that the signal from the slot-blots were generated from primary antibody binding to the target antigen.

Immunoblotting was carried out using mouse monoclonal antibodies against muscle-specific c-met (1:2,000-fold dilution; Cell Signaling Technology Inc., Manvers, MA) and PCNA (1:2,000-fold dilution; Cell Signaling Technology Inc.). Immunodetection of muscle [IGF-1] was performed using competitive EIA kits (DRG International, Mountainside, NJ). For muscle [c-met], nonspecific binding sites on the nitrocellulose membranes were blocked with NFBA for 50 minutes at room temperature. Membranes were then incubated with one of the aforementioned primary antibody solutions (fold-diluted antibody in NFBA) for 1-2 hours at room temperature. For the c-

met slot blots, membranes were incubated with the primary antibody solution at 4 C overnight in order to increase antigen binding. The primary antibody solution was then decanted, membranes were washed two times at 5 minutes per wash with TBST, and membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody solution (20,000-fold dilution in TBST; Bio-Rad Laboratories) for 45 minutes. Finally, the secondary antibody solution was decanted, membranes were washed three times at 5 minutes per wash with TBST, and membranes were incubated with a an enhanced chemiluminescent reagent (Bio-Rad Laboratories) for 5 minutes. Membranes were then placed in the gel documentation system (Bio-Rad Laboratories), and immunoprobed band densities were determined using band densitometry using Quantity One software (Bio-Rad Laboratories). The exposure times for each antigen were as follows: 2 minutes for PCNA Western blots and 2 minutes for c-met slot-blots. For c-met slot blots, membranes were initially probed with primary antibody, stripped in Western blotting stripping buffer for 5 minutes, and reprobed with the 2' antibody solution only in order to ensure that there was not a false signal generated by non-specific binding of the secondary antibody solution. All muscle [PCNA] and [c-met] data were expressed as band density/Ponceau density. Muscle [IGF-1] was expressed as ng IGF-1/mg protein. Coefficient of variation values between duplicates were: PCNA = 10.4%, c-met = 8.8%, IGF-1 = 1.7%.

Statistical analyses

Due to the large variances commonly attributed with molecular variables (i.e., inter-individual differences in DNA, mRNA, and protein concentrations), the Shapiro-Wilk statistic was performed for each dependent variable at each time point to ensure a

normality in distribution existed. For normally distributed molecular data, a two-way [age (young vs old males) x testing session (T1 biopsy, pre-T2 biopsy, pre-T3 biopsy, T4 biopsy)] ANOVA with repeated measures was used to determine main and interactive effects. Further, selected data that are normally distributed was correlated using the Pearson's product-moment correlation. For dependent variables that exhibited a non-normal distribution (i.e., skewness and/or kurtosis > 2.0, or Shapiro-Wilk statistic $p < 0.05$) then non-parametric statistics were performed. Specifically, the Mann-Whitney U statistic was used to determine which condition(s) were significantly different at each time point for each non-normally distributed dependent variable (synonymous to an independent 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 is < 0.05 then Wilcoxin signed rank tests were employed as a *post hoc* measure to determine which time points were significantly different within both age groups (via the split file function). Finally, selected variables that are non-normally distributed were correlated using the Spearman's rank correlation. An alpha of $p \leq 0.05$ was used to determine significance for all statistical tests, and all analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

CHAPTER IV

RESULTS

Participant Demographics, Dietary Intakes, and Lifting Comparisons

Ten healthy younger males [means ± SE; age: 21.0 ± 0.5 years, body mass: 82.3 ± 4.2 kg, height: 178.4 ± 2.2 cm, body fat percentage (3-site SKF: chest, abdomen, thigh): 15.4 ± 2.9%] and ten healthy older males [age: 66.4 ± 1.6 years, body mass: 94.2 ± 3.7 kg, height: 180.9 ± 2.2 cm, body fat percentage (3-site SKF: chest, abdomen, thigh): 27.4 ± 1.8%] volunteered to participate in this study. According to 2-3 day food logs, caloric intake (young = 33.4 ± 5.1 kcal/kg/d, old = 19.5 ± 1.9 kcal/kg/d; $p = 0.038$), protein intake (young = 1.6 ± 0.2 g/kg/d, old = 0.8 ± 0.1 g/kg/d; $p = 0.030$), carbohydrate intake (young = 4.1 ± 0.6 g/kg/d, old = 2.3 ± 0.3 g/kg/d; $p = 0.017$), and fat intake (young = 1.2 ± 0.2 g/kg/d, old = 0.6 ± 0.1 g/kg/d; $p = 0.006$) were all greater in the young versus old males. Between-age strength comparisons using independent samples t-tests indicated that the younger participants exhibited greater 1RMs for hack squat (young: 170.0 ± 10.9 kg, old: 105.8 ± 10.4 kg; $p = 0.001$), leg press (young: 271.1 ± 14.6 kg, old: 182.7 ± 14.9 kg; $p < 0.001$), leg extension (young: 64.8 ± 4.0 kg, old: 46.7 ± 2.8 kg; $p = 0.002$), as well as the cumulative training volume for the T1-T3 workouts (young: 36,392 ± 1,894 kg, old: 23,724 ± 1,639 kg; $p < 0.001$).

Normality Distribution of Dependent Variables

As mentioned, the Shapiro-Wilk statistic was performed for each dependent variable at each time point to ensure a normality in distribution existed (Table 3). Briefly, muscle [total RNA], muscle [DNA], $P21^{CIP1}$ mRNA expression, and $P27^{KIP1}$

mRNA expression values were normally distributed across all time points and were analyzed using parametric statistics. The other dependent variables including muscle [total protein], muscle [myofibrillar protein], *MGF* mRNA expression, *CDK2/4* mRNA expression, *CYCLIN D1* mRNA expression, *IGF-1EA* mRNA expression, *MGF:IGF-1EA* mRNA, MHC_{EMB} mRNA expression, and *MYOD* mRNA expression values were analyzed using non-parametric statistics.

Muscle [DNA]

Muscle [DNA] values are presented in Figure 7. For the young group, muscle [DNA] values (ng/mg wet muscle) expressed as mean ± SE were: T1 = 346 ± 39, T2 = 283 ± 37, T3 = 267 ± 44, T4 = 285 ± 27. For the old group, muscle [DNA] values (ng/mg wet muscle) expressed as mean ± SE were: T1 = 336 ± 57, T2 = 318 ± 82, T3 = 274 ± 30, T4 = 289 ± 33. A mixed factorial (2 age groups x 4 time point) ANOVA indicated that there was no main group effect ($p = 0.88$), no main time effect ($p = 0.07$), and/or no age x time point interaction ($p = 0.78$).

Muscle [Total Protein] and [Myofibrillar Protein]

Muscle [total protein] values are presented in Figure 8. For the young group, muscle [total protein] values (μg/mg wet muscle) expressed as mean ± SE were: T1 = 81 ± 11, T2 = 87 ± 11, T3 = 91 ± 9, T4 = 84 ± 7. For the old group, [total protein] values (μg/mg wet muscle) expressed as mean ± SE were: T1 = 75 ± 5, T2 = 87 ± 12, T3 = 68 ± 5, T4 = 79 ± 10. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that muscle [total protein] values were not different

between age groups (p-values: T1 = 0.97, T2 = 0.91, T3 = 0.11, T4 = 0.53). A Friedman test indicated that there was no main effect for time regarding muscle [total protein] values (p = 0.58).

Muscle [myofibrillar protein] values are presented in Figure 9. For the young group, muscle [myofibrillar protein] values (μg/mg wet muscle) expressed as mean ± SE were: T1 = 16 ± 2, T2 = 14 ± 1, T3 = 12 ± 1, T4 = 13 ± 1. For the old group, [myofibrillar protein] values (μg/mg wet muscle) expressed as mean ± SE were: T1 = 14 ± 1, T2 = 13 ± 1, T3 = 12 ± 1, T4 = 13 ± 1. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that muscle [total protein] values were not different between age groups (p-values: T1 = 0.55, T2 = 0.91, T3 = 0.39, T4 = 0.74). A Friedman test indicated that there was no main effect for time regarding muscle [myofibrillar protein] values (p = 0.14).

Muscle [Total RNA]

Muscle [total RNA] values are presented in Figure 10. For the young group, muscle [total RNA] values (ng/mg wet muscle) expressed as mean ± SE were: T1 = 379 ± 28, T2 = 328 ± 27, T3 = 427 ± 38, T4 = 342 ± 35. For the old group, muscle [total RNA] values (ng/mg wet muscle) expressed as mean ± SE were: T1 = 387 ± 30, T2 = 333 ± 34, T3 = 434 ± 46, T4 = 332 ± 49. A mixed factorial (2 age groups x 4 time point) ANOVA indicated that there was no main group effect (p = 0.99) nor was there an age x time point interaction (p = 0.78), although there was a main time effect (p = 0.002). As a follow-up analysis to the significant main time effect, within-group dependent t-tests indicated that muscle [total RNA] increased within the young (p = 0.021) and old (p =

0.010) groups from T3 to T4. Conversely, there was also a significant decrease in muscle [total RNA] within the old group (p = 0.021) groups from T3 to T4. There were no other within-group changes in muscle [total RNA] over the course of the exercise intervention.

Pre- and Post-Exercise Expression of Genes of Interest

CDK2 mRNA expression values are presented in Figure 12. For the young group, *CDK2* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00015965 ± 0.00003109, T2 = 0.00021074 ± 0.00004638, T3 = 0.00023999 ± 0.00007926, T4 = 0.00016971 ± 0.00003644. For the old group, *CDK2* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00035216 ± 0.00015678, T2 = 0.00021124 ± 0.00004691, T3 = 0.00019293 ± 0.00003765, T4 = 0.00026626 ± 0.00007150. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *CDK2* mRNA expression values were not different between age groups (p-values: T1 = 0.60, T2 = 1.00, T3 = 0.97, T4 = 0.32). A Friedman test indicated that there was no main time effect regarding *CDK2* mRNA expression values (p = 0.71).

CDK4 mRNA expression values are presented in Figure 13. For the young group, *CDK4* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00017246 ± 0.00002463, T2 = 0.00020397 ± 0.00003755, T3 = 0.00014755 ± 0.00002140, T4 = 0.00019044 ± 0.00003366. For the old group, *CDK4* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00017843 ± 0.00004542, T2 = 0.00014986 ± 0.00002405, T3 = 0.00017713 ± 0.00005429, T4 = 0.00018076 ± 0.00004162. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *CDK4* mRNA expression

values were not different between age groups (p-values: T1 = 0.80, T2 = 0.39, T3 = 0.85, T4 = 0.63). A Friedman test indicated that there was no main time effect regarding *CDK4* mRNA expression values (p = 0.60).

CYCLIN D1 mRNA expression values are presented in Figure 14. For the young group, *CYCLIN D1* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00002007 ± 0.00000505, T2 = 0.00003026 ± 0.00001091, T3 = 0.00004011 ± 0.00001927, T4 = 0.00002659 ± 0.00000871. For the old group, *CYCLIN D1* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00006085 ± 0.00001868, T2 = 0.00006297 ± 0.00000899, T3 = 0.00007353 ± 0.00002029, T4 = 0.00005950 ± 0.00001850. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *CYCLIN D1* mRNA expression values were significantly greater at T1 (p = 0.017) and T2 (p = 0.017) and tended to be greater at T3 (p = 0.063) and T4 (0.052) older versus younger participants. A Friedman test indicated that there was no main time effect regarding *CYCLIN D1* mRNA expression values (p = 0.56).

IGF-1EA mRNA expression values are presented in Figure 15. For the young group, *IGF-1EA* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00047902 ± 0.0000887, T2 = 0.00056631 ± 0.00004675, T3 = 0.00057156 ± 0.00010560, T4 = 0.00055007 ± 0.00011956. For the old group, *IGF-1EA* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00044641 ± 0.00004274, T2 = 0.00064208 ± 0.00011577, T3 = 0.00059338 ± 0.00008754, T4 = 0.00058086 ± 0.00015185. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *IGF-1EA* mRNA

expression values were not different between age groups (p-values: T1 = 0.68, T2 = 0.97, T3 = 0.50, T4 = 0.97). A Friedman test indicated that there was no main time effect regarding muscle *IGF-1EA* mRNA expression values (p = 0.076).

MGF mRNA expression values are presented in Figure 11. For the young group, *MGF* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00000337 ± 0.00000132, T2 = 0.00000474 ± 0.00000083, T3 = 0.00000562 ± 0.00000156, T4 = 0.00000577 ± 0.00000168. For the old group, *MGF* mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00000218 ± 0.00000038, T2 = 0.00000495 ± 0.00000106, T3 = 0.00000498 ± 0.00000119, T4 = 0.00000687 ± 0.00000209. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *MGF* mRNA expression values were not different between age groups (p-values: T1 = 0.91, T2 = 0.91, T3 = 0.97, T4 = 0.74). A Friedman test indicated that there was a main time effect regarding muscle *MGF* mRNA expression values (p = 0.017). As a follow-up analysis to the significant main time effect, within-group Wilcoxin signed rank tests indicated that *MGF* mRNA expression increased at T2 (p = 0.047), T3 (p = 0.037), and T4 (p = 0.022) compared to T1 within the old group. There were no within-group changes from baseline values in *MGF* mRNA expression within the young group throughout the duration of the exercise intervention (p > 0.05).

MGF:IGF-1EA mRNA expression values are presented in Figure 16. For the young group, *MGF:IGF-1EA* mRNA expression values expressed as mean ± SE were: T1 = 0.0059133 ± 0.0011131, T2 = 0.0085498 ± 0.0009540, T3 = 0.0099931 ± 0.0019179, T4 = 0.0162566 ± 0.0067148. For the old group, *MGF:IGF-1EA* mRNA

expression values expressed as mean ± SE were: T1 = 0.0049602 ± 0.0008362, T2 = 0.0075066 ± 0.0011970, T3 = 0.0075284 ± 0.0010465, T4 = 0.0109819 ± 0.0012423. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *MGF:IGF-1EA* mRNA expression values were not different between age groups (p-values: T1 = 0.74, T2 = 0.55, T3 = 0.40, T4 = 0.80). A Friedman test indicated that there was a main time effect regarding muscle *MGF:IGF-1EA* mRNA expression values (p = 0.003). As a follow-up analysis to the significant main time effect, within-group Wilcoxin signed rank tests indicated that *MGF:IGF-1EA* mRNA expression increased at T3 (p = 0.028), and T4 (p = 0.005) compared to T1 in the old group. There were no within-group changes from baseline values in *MGF:IGF-1EA* mRNA expression within the young group throughout the duration of the exercise intervention (p > 0.05).

MHC_{EMB} mRNA expression values are presented in Figure 17. For the young group, MHC_{EMB} mRNA expression values expressed as mean ± SE were: T1 = 0.00006773 ± 0.00001301, T2 = 0.00026983 ± 0.00010801, T3 = 0.00008849 ± 0.00002592, T4 = 0.00011329 ± 0.00002316. For the old group, MHC_{EMB} mRNA expression values expressed as mean ± SE were: T1 = 0.00008484 ± 0.00003740, T2 = 0.00011562 ± 0.00002667, T3 = 0.00045068 ± 0.00021586, T4 = 0.00022454 ± 0.00008955. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that MHC_{EMB} mRNA expression values were not different between age groups (p-values: T1 = 0.50, T2 = 0.53, T3 = 0.28, T4 = 0.97). A Friedman test indicated that there was a main time effect regarding muscle MHC_{EMB} mRNA expression values (p = 0.037). As a follow-up analysis to the significant main time

effect, within-group Wilcoxin signed rank tests indicated that MHC_{EMB} mRNA expression increased at T4 ($p = 0.011$) compared to T1 in the young group. There were no within-group changes from baseline values in MHC_{EMB} mRNA expression within the old group throughout the duration of the exercise intervention ($p > 0.05$).

MYOD mRNA expression values are presented in Figure 18. For the young group, *MYOD* mRNA expression values expressed as mean ± SE were: T1 = 0.00015993 ± 0.00002750, T2 = 0.00022781 ± 0.00002890, T3 = 0.00021403 ± 0.00005122, T4 = 0.00016172 ± 0.00002386. For the old group, *MYOD* mRNA expression values expressed as mean ± SE were: T1 = 0.00022134 ± 0.00003507, T2 = 0.00028401 ± 0.00006474, T3 = 0.00021536 ± 0.00004405, T4 = 0.00018959 ± 0.00006055. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that *MYOD* mRNA expression values were not different between age groups (p-values: T1 = 0.19, T2 = 0.85, T3 = 0.80, T4 = 0.53). A Friedman test indicated that there was a main time effect regarding muscle *MYOD* mRNA expression values ($p = 0.006$). As a follow-up analysis to the significant main time effect, within-group Wilcoxin signed rank tests indicated that *MYOD* mRNA expression increased at T2 ($p = 0.013$) compared to T1 in the young group. There were no within-group changes from baseline values in *MYOD* mRNA expression within the old group throughout the duration of the exercise intervention ($p > 0.05$).

$P21^{CIP1}$ mRNA expression values are presented in Figure 19. For the young group, $P21^{CIP1}$ mRNA expression values expressed as mean ± SE were: T1 = 0.00038837 ± 0.00016837, T2 = 0.00022784 ± 0.00004659, T3 = 0.00025679 ± 0.00003385, T4 = 0.00010424 ± 0.00001771. For the old group, $P21^{CIP1}$ mRNA expression values

expressed as mean ± SE were: T1 = 0.00033576 ± 0.00012319, T2 = 0.00035345 ± 0.00005194, T3 = 0.00031180 ± 0.00005617, T4 = 0.00023953 ± 0.00002607. A two-way (age x session) ANOVA employing the Huynh-Feldt indicated that there was no time effect (p = 0.13), group effect (p = 0.42), or interaction effect (p = 0.37) for $P21^{CIP1}$ mRNA expression values.

$P27^{KIP1}$ mRNA expression values are presented in Figure 20. For the young group, $P27^{KIP1}$ mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00074520 ± 0.00009994, T2 = 0.00088720 ± 0.00011419, T3 = 0.00075993 ± 0.00013020, T4 = 0.00077444 ± 0.00011908. For the old group, $P27^{KIP1}$ mRNA expression values (relative to *B2M & 28S* avg) expressed as mean ± SE were: T1 = 0.00100762 ± 0.00018271, T2 = 0.00087315 ± 0.00016167, T3 = 0.00081495 ± 0.00015854, T4 = 0.00064139 ± 0.00014644. A two-way (age x session) ANOVA indicated that there was no time effect (p = 0.28), group effect (p = 0.79), or interaction effect (p = 0.28) for $P27^{KIP1}$ mRNA expression values.

Muscle [PCNA], [c-met] and [IGF-1]

Muscle [PCNA] values are presented in Figure 21. For the young group, muscle [PCNA] expressed in arbitrary density units (ADUs) as mean ± SE were: T1 = 0.020 ± 0.007, T2 = 0.027 ± 0.008, T3 = 0.024 ± 0.007, T4 = 0.024 ± 0.007. For the old group, muscle [PCNA] expressed in ADUs as expressed as mean ± SE were: T1 = 0.018 ± 0.006, T2 = 0.019 ± 0.005, T3 = 0.019 ± 0.005, T4 = 0.019 ± 0.005. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that muscle [PCNA] values were not different between age groups (p-values: T1 = 0.74, T2 =

0.85, T3 = 0.85, T4 = 0.97). A Friedman test indicated that there was not a main time effect regarding muscle [PCNA] values ($p = 0.44$).

Muscle [c-met] values are presented in Figure 22. For the young group, muscle [PCNA] expressed in arbitrary density units (ADUs) as mean ± SE were: T1 = 1.17 ± 0.12, T2 = 1.42 ± 0.14, T3 = 0.90 ± 0.13, T4 = 0.72 ± 0.06. For the old group, muscle [c-met] expressed in ADUs as expressed as mean ± SE were: T1 = 1.01 ± 0.21, T2 = 1.23 ± 0.13, T3 = 1.05 ± 0.11, T4 = 0.97 ± 0.12. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that muscle [c-met] values were not different between age groups (p-values: T1 = 0.14, T2 = 0.48, T3 = 0.48, T4 = 0.11). A Friedman test indicated that there was a main time effect regarding muscle [c-met] values ($p < 0.001$). As a follow-up analysis to the significant main time effect, within-group Wilcoxin signed rank tests indicated that muscle [c-met] values increased at T2 ($p = 0.005$) and decreased at T4 ($p = 0.022$) compared to T1 in the young group. There were no within-group changes from baseline values in muscle [c-met] values within the old group throughout the duration of the exercise intervention ($p > 0.05$).

Muscle [IGF-1] values are presented in Figure 23. For the young group, muscle [IGF-1] expressed in ng/mg skeletal muscle as mean ± SE were: T1 = 0.195 ± 0.153, T2 = 0.058 ± 0.022, T3 = 0.215 ± 0.173, T4 = 0.166 ± 0.112. For the old group, muscle [IGF-1] expressed in ng/mg skeletal muscle as mean ± SE were: T1 = 0.219 ± 0.159, T2 = 0.158 ± 0.120, T3 = 0.234 ± 0.196, T4 = 0.439 ± 0.245. Between-group mean comparisons at each time point using separate Mann Whitney U tests indicated that muscle [IGF-1] values were not different between age groups (p-values: T1 = 0.85, T2 =

0.58, T3 = 0.53, T4 = 0.63). A Friedman test indicated that there was no main time effect regarding muscle [IGF-1] values ($p = 0.80$).

CHAPTER V

DISCUSSION

Mechanistic studies such as the current investigation are of critical importance due to the fact that the hypertrophic potential to resistance exercise has been shown to be unexplainably impaired with aging [9, 15, 121], albeit other evidence suggests that resistance exercise-induced muscle hypertrophy is similar between younger and older adults [122]. In this regard, studies such as the current investigation allow scientists to better understand the etiology behind muscle aging. This study specifically outlined the age-related differences in the expression of genes and proteins related to satellite cell activity prior to and following three conventional bouts of resistance exercise. Given that past research has indicated that: 1) the satellite cell proliferative potential is impaired in older versus younger males at rest [3] and in response to weeks of resistance exercise [15], and 2) satellite cell proliferation impairments are causally linked to long-term increases in muscle mass [14], our laboratory was interested in determining if age-related impairments in myogenic genes and/or growth factors existed prior to and during one week of resistance training. In the current study, three sequential bouts of conventional lower body resistance training increased muscle [c-met], a protein marker indicative of satellite cell quantity, in young males 48 hours following the first bout ($p < 0.01$) and decreased this marker in young males 24 hours following the third bout ($p < 0.05$), whereas this value did not change within the old group. Likewise, *MYOD* gene expression modestly increased 48 hours following the first bout ($p < 0.05$) and MHC_{EMB} gene expression modestly increased 24 hours following the third bout ($p < 0.05$) relative to baseline expression values in the young males ($p < 0.05$). Interestingly, *MGF*

expression increased at T2-4 in the older group relative to baseline values ($p < 0.05$) which is contrary to other reports examining this gene at different biopsy time points. Taking all of these findings into consideration indicate that repeated training bouts: 1) may increase satellite cell number in younger males following the first initial bout as evidenced with the current muscle [c-met] data, 2) do not alter the expression of genes indicative of satellite cell activity (i.e., *CDK2*, *CDK4*, *CYCLIN D1*, *MYOD*, $P21^{CIP1}$, $P27^{KIP1}$) and/or the expression of *IGF-1EA* up to 24-48 hours following exercise in younger or older males, 3) do not increase other markers of satellite cell proliferation (i.e., muscle [PCNA], and [DNA]) in either age group at the sampled time points, and 4) do not alter [myofibrillar protein] or [total protein] in either age group at the sampled time points. In regards to the pre-intervention findings, *CYCLIN D1* mRNA was more highly expressed in older versus younger males ($p < 0.05$), whereas all other genes and markers of satellite cell activity were similar between age groups. Therefore, these data may also suggest that the proliferative potential of satellite cells is sustained during resting, un-exercised states in aging males. Note that 3-day food logs revealed that caloric and macronutrient intakes relative to body mass were also found to be greater in the young versus old group. Interestingly, researchers that have reduced protein intakes (i.e., 0.5 g/kg/d) over a one-week period in 50-80 year-old males demonstrated that inadequate Calorie and/or protein intake adversely affects the skeletal muscle transcriptome [123]. These authors stated:

> *"The results of this study show that older persons who consume inadequate dietary protein for 1 wk experience a wide variety of changes in transcript levels in skeletal muscle. These responses are consistent with the existence of metabolic*

and physiologic changes that may precede accommodation, including inflammatory stress, reduced metabolism and oxygen transport, reduced protein synthesis, and muscle wasting."

Nonetheless, our current method of dietary assessment using 3-day recalls contains methodological limitations (i.e., under-reporting which is common with these methods as well as a limited n-size) and precludes us from making inferences as to how dietary variables may affect the skeletal muscle environment in the current study.

Baseline Markers of Satellite Cell Activity between Age Groups

As mentioned, *CYCLIN D1* gene expression was significantly greater in older versus younger males ($p < 0.05$), whereas all other genes (i.e., *CDK2*, *CDK4*, MHC_{EMB}, *MYOD*, $P27^{KIP1}$, $P21^{CIP1}$) and markers of satellite cell activity (i.e., muscle [DNA], muscle [PCNA], and muscle [c-met]) were similar between age groups. Contrary to the initial hypotheses, these data seemingly suggest that the proliferative states of satellite cells are sustained during resting, un-exercised states in aging males versus younger counterparts. This contention, however, possesses methodologically limitations due to the fact that fiber type-specific satellite cell numbers were not microscopically examined. In this regard, Verdijk et al. [3] used immunohistochemistry to double-label adjacent muscle biopsy cross sections and determined that the number of type II fiber satellite cells were approximately 50% lower in 76 year old versus 20 year old males. However, other researchers have determined that satellite cell number in the human vastus lateralis muscle does not change with aging [15, 16, 118, 124]. Assuming that satellite cells maintain a constant expression pattern of c-met and due to the fact that this protein was

quantified from the lysate of a large, representative muscle sample in the current study provides evidence to suggest that satellite cell numbers are indeed similar between younger and older males. In order to support this finding, future attempts will be made by our laboratory to quantify satellite cell numbers in these participants using immunohistochemistry.

The finding that most of the investigated cell cycle-regulating genes (i.e., *CDK2*, *CDK4*, *MYOD*, $P21^{CIP1}$, and $P27^{KIP1}$) were similar between younger and older participants is in agreement with some investigations and dissimilar with others. For instance, the intramuscular protein expression of cyclin D1 and $p27^{Kip1}$ have not been affected by human aging [18]. Conversely, Raue et al. [91] reported that the myogenic expression of *MYOD* mRNA is 58% greater in older versus younger women. Recent rodent data has demonstrated a 50% decrement in $p27^{Kip1}$ protein levels from older cultured rat satellite cells [19], and similar data has reported a decrement in the genetic expression of $P57^{KIP2}$ and $P21^{CIP1}$ from the skeletal muscle of aged mice; an effect which may contribute to a diminished capability of satellite cells to differentiate [30]. One explanation for the divergent findings between the current and aforementioned studies is due to the fact that baseline gene expression was normalized to the arithmetic average of two housekeeping genes which have been shown to not be affected by aging. In this regard, Raue et al. normalized the reported *MYOD* data to *GAPDH*, which we have found to be expressed more highly (+100%) in younger participants. Therefore, normalizing *MYOD* mRNA expression values to *GAPDH*, which decreases with aging, will greatly inflate these data in older participants. The increased mRNA expression of *CYCLIN D1* with aging may be a compensatory mechanism whereby satellite cells within older

skeletal muscle are attempting to sustain proliferative capabilities. In support of this hypothesis, a five-week heterochronic parabiosis experiment (or the pairing of the circulatory systems of young and old animals) followed by muscle injury demonstrated that the old muscle from heterochronic pairing exhibited a substantially greater number of MHC_{EMB}-positive cells (indicative of satellite cell proliferation and differentiation) compared to the older muscles in isochronic pairings [107]. In their concluding remarks, Conboy et al. stated,

> *Together, these in vivo and in vitro data demonstrate that components in young serum alone are capable of reversing the molecular and cellular aspects of age-related decline in muscle stem cell activation. In addition, it also seems that factors in old serum negatively affect the processes required for satellite cell activation and muscle repair.*

Contextually applying these data to the current study may indicate that the endocrine and/or paracrine environments in older humans quells satellite cell activity; an effect which obviates the need for an increased expression of proliferative genes (such as *CYCLIN D1*) in order to maintain satellite cell populations.

Baseline *MGF* and *IGF-1EA* Gene and IGF-1 Protein Expression between Age Groups

The current data demonstrate that *MGF* and *IGF-1EA* mRNA expression patterns remain stable with aging in resting, unexercised states which is in partial or full agreement with numerous investigations. Haddad and Adams [22] recently determined that resting MGF mRNA expression was similar between young and old rodents, albeit *IGF-1EA* transcripts were more highly expressed in old rodents. However, the current

findings are in agreement with Hameed et al. [10] as well as Petrella et al. [15] who determined that *MGF* and *IGF-1EA* mRNA is similarly expressed in the skeletal muscle of young and old humans.

Muscle [IGF-1] peptide concentrations also remain stable with aging in resting, unexercised states which, to our knowledge, is a novel finding in humans. Haddad and Adams [22] recently demonstrated that IGF-1 receptor concentrations are significantly greater in older versus younger rodents, albeit insulin receptor substrate-1 (IRS-1, both total and phosphorylated/activated levels) as well as phosphorylated Akt concentrations were depressed in older versus younger rodents. Simply stated, our findings illustrate that: 1) IGF-1 peptide expression is minimally affected with aging and 2) a molecular disconnect between IGF-1receptor signaling in skeletal muscle (i.e., the IGF-1 receptor-mediated activation of IRS-1) may be of further relevance in regards to the development of sarcopenia.

Myogenic Gene Expression Patterns and Muscle [IGF-1] Following Resistance Exercise

The lack of change in the current post-exercise *CDK2*, *CDK4*, *CYCLIN D1*, $P21^{CIP1}$, and $P27^{KIP1}$ mRNA expression data may be explained by the effects of resistance training on the transient (i.e., within 24 hours) versus the prolonged expression (i.e., 48 hours post-exercise) patterns. For instance, a plethora of evidence exists suggesting that myogenic gene expression occurs transiently (i.e., within 24 hours) in response to mechanical loading. Psilander et al. [57] demonstrated that *MYOGENIN*, *MYOD*, and *MRF4* mRNA levels were elevated in human skeletal muscle by 100-400% at 0, 1, 2, 6, and 24 hours following a leg press protocol consisting of 4 sets of 6-12

repetitions. Likewise, a significant 4-hour post-exercise increase in *MYOD* mRNA (2.0-fold) has also been reported to occur in younger and older women [91], and a 12-24-hour post-exercise increase in *MYOD* mRNA (+83%), *CYCLIN D1* mRNA (+50%), and $P21^{CIP1}$ mRNA (+1,600%) has been reported to occur in younger males and females [29]. Bamman et al. [17] also reported that $P27^{KIP1}$ mRNA modestly decreased (-16%) and *CYCLIN D1* mRNA modestly increased (+34%) in younger and older males and females 24 hours following resistance exercise, and our laboratory has found that one bout of lower body resistance training increased *CDK4* expression 6 hours post-exercise (+60%), decreased *MYOD* expression 6 hours post-exercise (-80%), decreased $P27^{KIP1}$ expression 6 hours post-exercise (-640%), and substantially increased $P21^{CIP1}$ expression 2- and 6 hours post-exercise (+1,250% and +4,670%, respectively) in younger males [125]. Thus, the mRNA expression patterns of *CDK2*, *CDK4*, *CYCLIN D1*, $P21^{CIP1}$, and $P27^{KIP1}$ are seemingly responsive to mechanical loading within a transient post-exercise window which was not captured in the current investigation. Furthermore, three sequential bouts of conventional resistance exercise appear to have no summative effects on the culmination of these genes in younger or older males.

As mentioned previously, *MGF* expression increased at T2 (+224%), T3 (+174%), and T4 (+290%) in the older group relative to baseline values ($p < 0.05$), and *IGF-IEA* mRNA expression patterns remained unaltered in both age groups; both of these findings being contrary to other reports examining these genes at different biopsy time points. Hameed et al. [10] determined that *MGF* mRNA expression substantially increased in younger (+2 – +864%) but not older subjects 2.5 hours following resistance exercise. Similarly, Bamman et al. [17] determined that *MGF* mRNA increased (+49%)

in younger and older humans 24 hours following a single bout of conventional resistance exercise, albeit *post hoc* analysis revealed a significant increase in *MGF* after exercise in young men only (+91%). Equivocal evidence exists demonstrating that resistance exercise did not affect *MGF* mRNA expression in younger subjects up to 48 hours following exercise, whereas *IGF-1EA* mRNA content decreased transiently following exercise (-44% at 1 and 6 h post-exercise, respectively) [57]. Thus, the current data is the first to suggest that: 1) the myogenic expression of *MGF* mRNA may be upregulated in a delayed fashion following conventional resistance exercise in older versus younger adults, 2) *IGF-1EA* mRNA expression is minimally affected 24-48 hours following three exercise bouts in younger and older humans, and 3) repeated bouts of resistance exercise may lead to the culmination in *MGF* mRNA in older humans. If the latter findings hold true then there may be physiological ramifications of the delayed post-exercise *MGF* expression pattern in older skeletal muscle. For instance, protein synthesis rates are known to transiently peak within a 24-hour window following resistance exercise [126]. Hence, if the peak expression of *MGF* mRNA occurs when post-exercise protein synthesis rates return to baseline in older skeletal muscle, then MGF peptide translation may not occur at an appreciable level. Therefore, future investigations need to discern if an increase in skeletal muscle MGF peptide content occurs in younger and older muscle following repeated training bouts and, more importantly, how potential increases in this peptide are crucial for long-term resistance training adaptations.

Contrary to the aforementioned findings, *MYOD* (+60%) and MHC_{EMB} (+130%) mRNA expression significantly increased compared to baseline values in the younger participants at T2 and T4, respectively. In regards to the former finding, several of the

aforementioned investigations have demonstrated that *MYOD* mRNA expression increases in humans in response to resistance training [29, 57, 91]. Furthermore, post-exercise increases in the MyoD transcription factor have been shown to shadow increases in *MYOD* mRNA expression [93, 127]. The MyoD transcription factor is known to initiate myoblast differentiation as well as increase transcription of contractile, cytoskeletal and metabolic genes [127], albeit no study has demonstrated that differentiation occurs in response to one training session. Thus, the increase in *MYOD* mRNA within the young group is somewhat of a 'normal' myogenic response to resistance exercise that may lead to the eventual differentiation of satellite cells once the need for extra myonuclear domains is warranted. Conversely, the increase in MHC_{EMB} transcripts within the young group 24 hours following the third exercise bout is a novel finding. Our laboratory has previously determined that MHC_{EMB} mRNA expression does not increase in younger or older males 24 hours following a single lower body exercise bout [113]. However, the increase observed in the current study within the young group following the third exercise bout may indicate that a subpopulation of satellite cells may have begun differentiating at this time point [31]. In this regard, future immunohistochemistry studies (i.e., identifying MHC_{EMB}+ cells with fluorescently-tagged antibodies) mimicking the current study design should be employed to confirm these findings.

Interestingly, our results are the first to demonstrate that repeated conventional exercise bouts do not alter total muscle IGF-1 peptide concentrations in younger or older human skeletal muscle. This finding contradicts other data in the current investigation suggesting that *MGF* mRNA expression increased at T2 ($p = 0.047$), T3 ($p = 0.037$), and

T4 ($p = 0.022$) compared to T1 within the old group. Further, this data contradicts recent data demonstrating that muscle MGF and IGF-1Ea peptides significantly increased ~125% 2 days and the IGF-1Ea peptide increase was sustained ~150% 5 days following an eccentric leg extensor bout [128]. Note, however, that we demonstrated that IGF-1 peptides increased 28%, 464%, and 326% in the young group and 175%, 837%, and 685% in the old group during T2-T4, albeit the large inter-individual responses over the training period precluded these changes from being significant. Our findings are in statistically in agreement with O'Reilly et al. [55] who recently demonstrated that one bout of eccentric exercise did not alter muscle HGF peptide concentrations at 4 hours, 24 hours, 72 hours or 120 hours post-exercise. Interestingly, Haddad and Adams [22] similarly demonstrated that young rodents experienced an increase in IGF-1 receptor activation 24 hours following a simulated resistance exercise bout, whereas old rodents did not experience this phenomenon; with this increase in young rodents returning to baseline at 48 hours post-exercise. This report seems to suggest that post-exercise IGF-1 receptor ligation and potentially muscle IGF-1 peptide levels peak within a 24-hour window in mammals. Taking all of these findings into consideration suggest that either: 1) conventional resistance exercise transiently increases the autocrine expression of muscle IGF-1 peptides following exercise and the current investigation did not capture this response, or 2) repetitive conventional exercise bouts may be needed to increase the mRNA accumulation of IGF-1 mRNA variants which eventually leads to an increase in the anabolic IGF-1 peptide signal in the muscle bed. In this regard, future research should examine how chronic resistance training affects muscle IGF-1 mRNA variants and peptides as well as IGF-1 receptor ligation patterns in younger versus older males in

concert with the phenotypic adaptations that occur with chronic training. These data will determine the relative importance of IGF-1 signaling with the process of skeletal muscle hypertrophy as well as how aging affects these processes.

Muscle [DNA], [PCNA], and [c-met] Following Resistance Exercise

There were no statistical differences within or between age groups for muscle [DNA] prior to or throughout the exercise intervention. While the DNA quantification method has been used successfully in the past to track changes in DNA following impractical and extreme mechanical overloading protocols in rodents [60], the lack of statistical changes in muscle [DNA] throughout the conventional exercise intervention is likely due to the lack of assay sensitivity. For example, it has been shown that satellite cell myonuclei contribute to approximately 2% of the total muscle myonuclear pool, and that these cells form 4-8 cell aggregates from a quiescent parent cell 3 days following a muscle-damaging stimulus [129]. Assuming the unlikely chance that 100% of the satellite cells in every subject proliferated into 8-cell clusters (or increase up to 800% in cell number) during the one-week training period would yield an approximate 14% increase in muscle [DNA]. In accordance with the muscle [c-met] values, satellite cell number likely modestly increased in younger males after one training bout, which has been reported previously to occur [55], and these increases were not consistently detected using fluorometric DNA quantification methods.

Although muscle [c-met] increased in the young group at T2, muscle [PCNA] values remained unaltered in both age groups throughout the one-week intervention. During the S phase of the cell cycle, PCNA acts as a homotrimer which assists with

leading strand synthesis during DNA replication. Furthermore, in vitro work has determined that PCNA concentrations increase prior increases in cell number during satellite cell proliferation [69]. The lack of change in PCNA indicated that either: 1) the biopsying time points did not capture changes in PCNA when/if they occurred in the young group between T1 and T2, and/or 2) muscle PCNA did not change because satellite cell proliferation did not occur at other time points. This is a novel finding due to the fact that muscle PCNA protein expression is rarely examined in vivo settings. One recent study did examine the expression of muscle PCNA protein between healthy and liver-diseased rodents and reported that muscle [PCNA] was significantly lower in the latter group [130]. In lieu of their findings these authors stated, "*In the present study, lower PCNA levels in the gastrocnemius muscle of [diseased] rats suggest impaired function of satellite cells.*" Interestingly, Deldicque et al. [131] recently reported that *PCNA* mRNA significantly increased immediately and 72 hours following 10 sets of 10 leg extensor repetitions at 80% 1RM in young males, while returning to baseline levels 24 hours post-exercise. Therefore, if PCNA protein values also follow the abovementioned trend then it is likely that the biopsy time points in the current study did not capture PCNA protein responses to exercise.

As previously mentioned, muscle [c-met] initially increased at T2 and decreased below baseline values by T4 in the young group only. The former finding is in agreement with numerous investigations demonstrating that one bout of eccentric resistance exercise is capable of significantly increasing satellite cell number in young males one to five days following exercise [16, 55, 65]. Thus, while these findings need to be microscopically examined, it is plausible that one bout of conventional lower body

resistance exercise is capable of stimulating increases in satellite cell number in young males. The inability for muscle [c-met] to increase in the older participants at any point throughout the intervention is also in agreement with a previous study that demonstrated that satellite cell proliferation is quelled in older individuals 24 hours following a strenuous eccentric exercise bout [16]; specifically, young males exhibited a 141% increase in satellite cell number and old men exhibited a 51% increase. Therefore, due to the fact that conventional resistance exercise is not as damaging as eccentric exercise, the three sequential exercise bouts employed in the current study was not enough of a mechanical stimulus to activate satellite cells in older individuals.

The decrement in muscle [c-met] within the young group at T4 is more difficult to explain, albeit it does seem to follow a similar physiological pattern which has been reported by previous investigations examining satellite cell number following acute versus prolonged resistance training. For instance, acute resistance training bouts have been shown to increase satellite cell number (+150-200% [16, 55, 65]) much greater than prolonged training programs spanning 12-16 weeks (i.e., +46% [39] and +49% [15]) in young males. What is more compelling is data reported by O'Reilly et al. [55] demonstrating that satellite cell numbers peaked three days following a strenuous exercise bout in young men, and began returning back to baseline values 5 days post-exercise. Taking all of these data into consideration may suggest that satellite cell proliferation creates satellite cell numbers that exceed physiological needs following an initial exercise bout. Furthermore, a subpopulation of these 'unneeded' satellite cells may undergo apoptosis during more prolonged post-exercise time points bringing satellite cell numbers back down to baseline values. However, it is impossible to confirm this

hypothesis since no one has ever examined potential apoptotic activity in skeletal muscle in concert with microscopically examining satellite cell numbers during prolonged recovery periods following resistance exercise.

Conclusions

In summary, these data suggest that: 1) three consecutive bouts of resistance exercise seem to significantly increase a marker indicative of satellite cell quantity concomitant with the increased expression of a select few myogenic genes in younger participants only (i.e., *MYOD* at T2 and MHC_{EMB} at T4), 2) repeated exercise bouts facilitated a summation effect on *MGF* expression only in older individuals which is contrary to preliminary research examining this gene in these populations, and 3) both age groups retain proliferative capabilities during resting, unexercised states given the similarities between these groups in regards to muscle [PCNA], muscle [c-met], *CDK2*, *CDK4*, as well as an increased *CYCLIN D1* mRNA expression in older individuals. Taking all of these findings into consideration suggests that additional exercise bouts may be needed to stimulate satellite cell activity during the initial stages of resistance training in older populations, albeit future research should employ immunohistochemistry in order to confirm these preliminary findings. Furthermore, the reported decrements in satellite cell activity (as assessed by muscle [c-met]) may indicate that a greater impetus is needed to activate satellite cells and stimulate muscle hypertrophy. Nonetheless, the current findings suggest that the examined cell cycle regulator mRNAs as well as IGF-1 variants are similarly expressed at baseline and following exercise between age groups raising the possibility that other genes and/or the satellite cell niche are what cause inherent age-

related differences in satellite cell physiology in response to exercise. To this end, future research should determine how endogenous decrements in circulating factors (i.e., hormones, leukocytes, and growth factors), niche factors (i.e., intramuscular fibroblast and adipocyte signaling), and other intrinsic factors (i.e., the Notch signaling cascade as an example) contribute to decrements in satellite cell activity with aging. These data will enable clinicians to develop more effective therapies at combating age-related muscle loss above and beyond employing resistance training interventioned

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Appendix A

Figure 1.

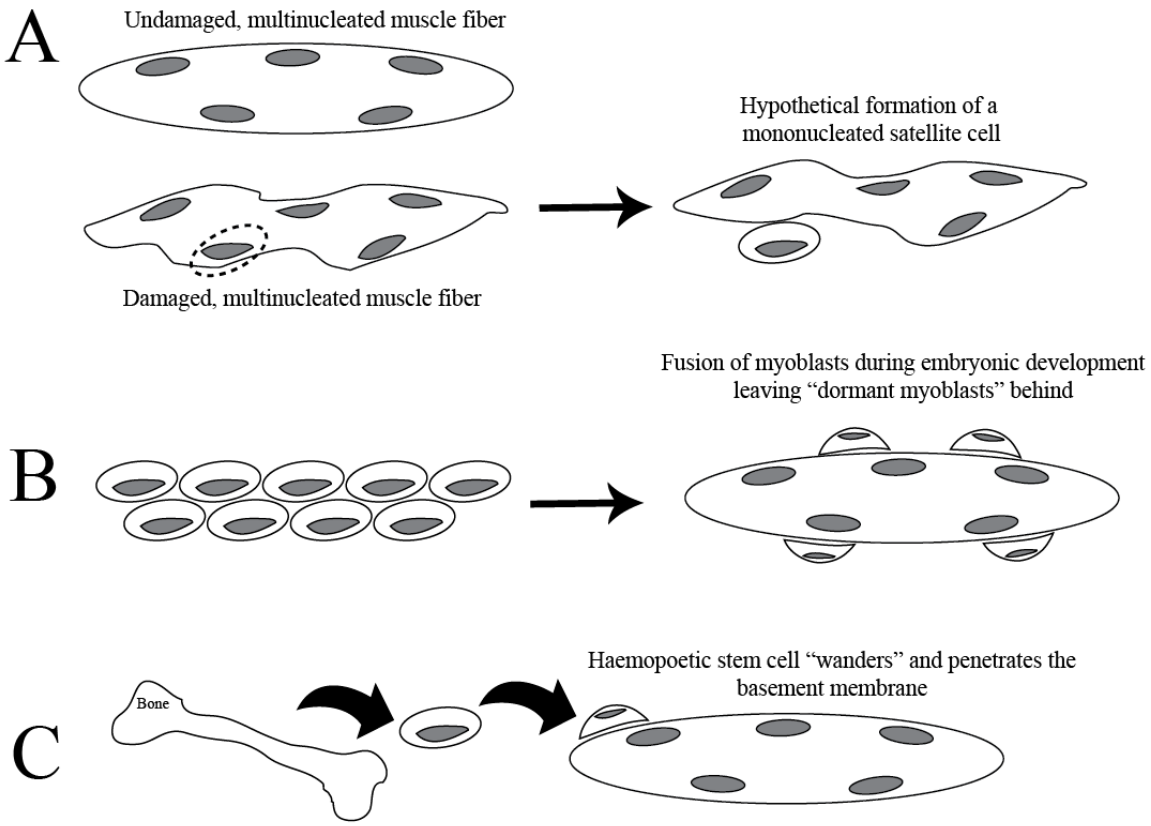


Figure 2.

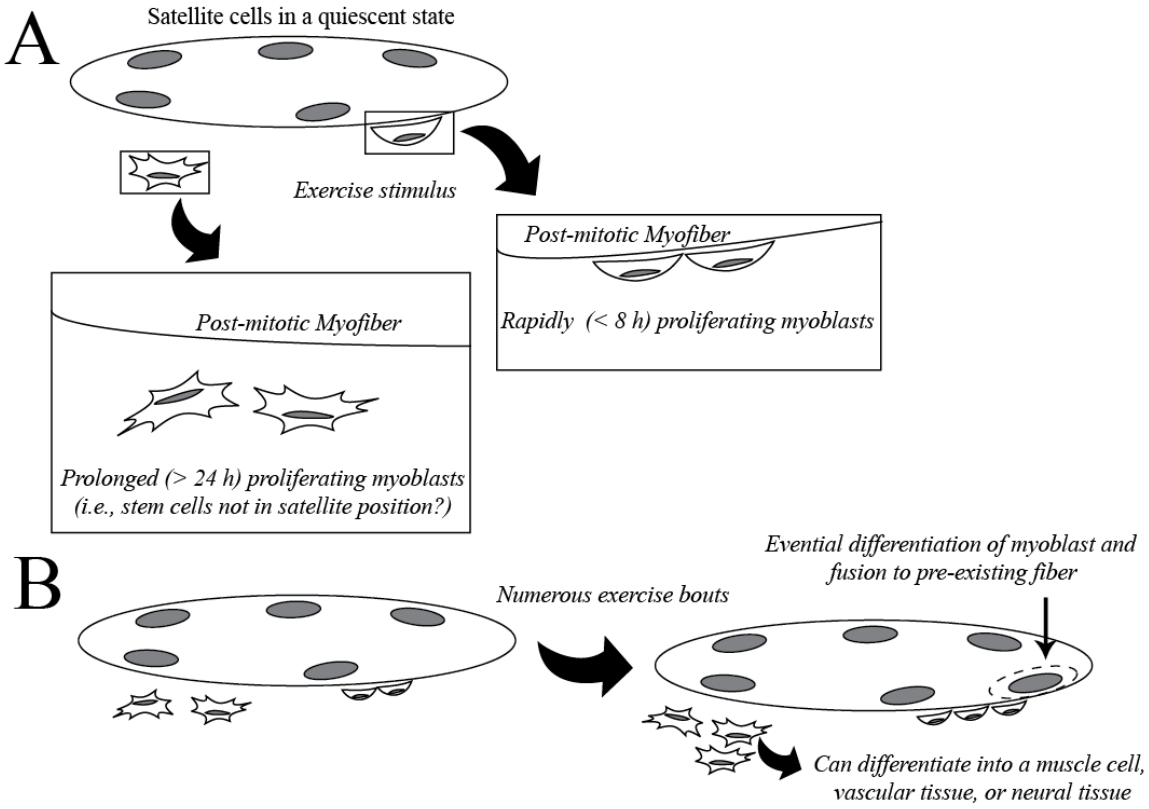

A
Satellite cells in a quiescent state
Exercise stimulus
Post-mitotic Myofiber
Rapidly (< 8 h) proliferating myoblasts
Post-mitotic Myofiber
Prolonged (> 24 h) proliferating myoblasts
(i.e., stem cells not in satellite position?)
B
Numerous exercise bouts
Evential differentiation of myoblast and
fusion to pre-existing fiber
Can differentiate into a muscle cell,
vascular tissue, or neural tissue

Figure 3.

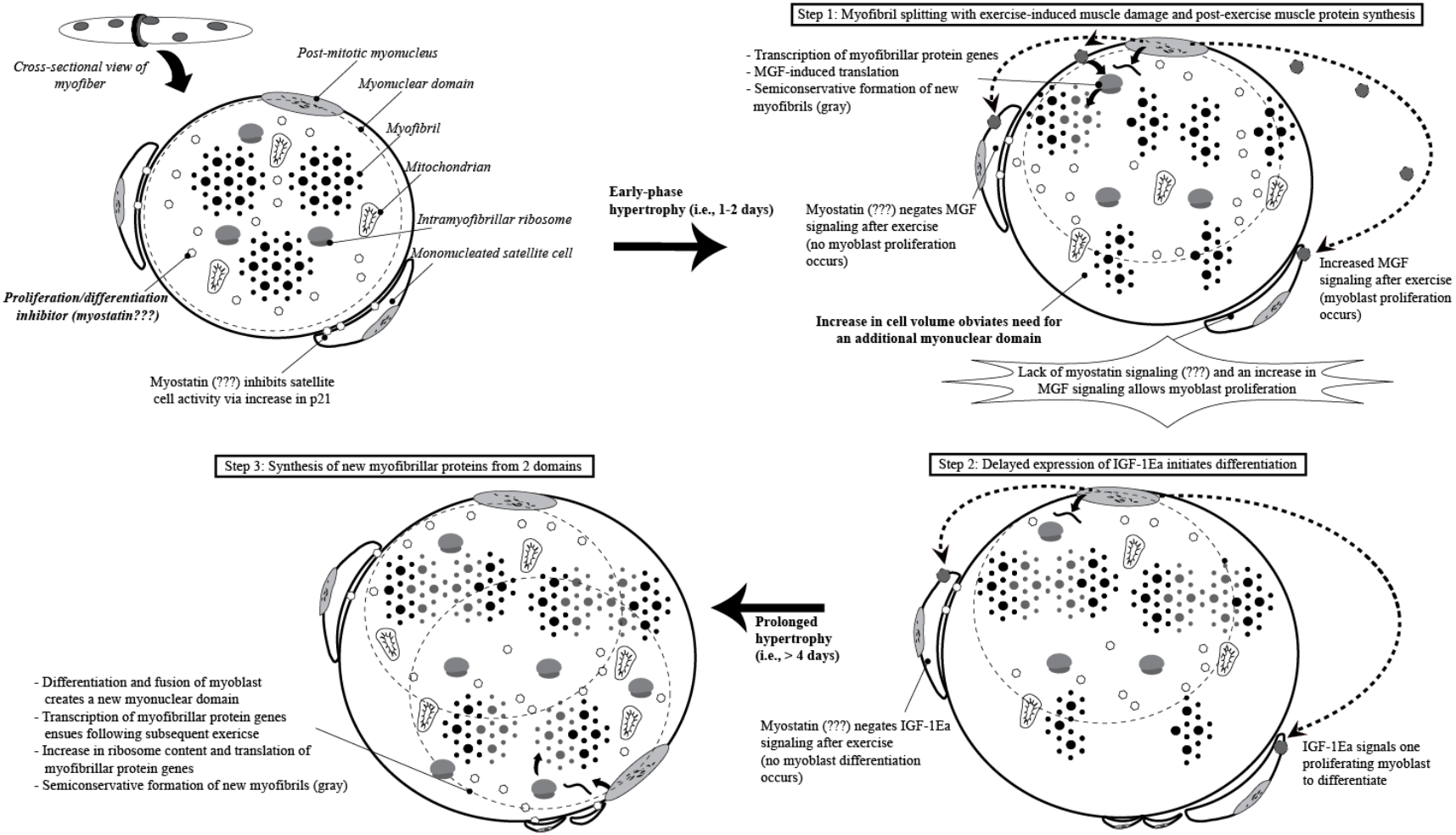

Cross-sectional view of myofiber
Post-mitotic myonucleus
Myonuclear domain
Myofibril
Mitochondrian
Intramyofibrillar ribosome
Mononucleated satellite cell
Proliferation/differentiation inhibitor (myostatin???)
Myostatin (???) inhibits satellite cell activity via increase in p21
Early-phase hypertrophy (i.e., 1-2 days)
Step 1: Myofibril splitting with exercise-induced muscle damage and post-exercise muscle protein synthesis
- Transcription of myofibrillar protein genes
- MGF-induced translation
- Semiconservative formation of new myofibrils (gray)
Myostatin (???) negates MGF signaling after exercise (no myoblast proliferation occurs)
Increased MGF signaling after exercise (myoblast proliferation occurs)
Increase in cell volume obviates need for an additional myonuclear domain
Lack of myostatin signaling (???) and an increase in MGF signaling allows myoblast proliferation
Step 2: Delayed expression of IGF-1Ea initiates differentiation
Prolonged hypertrophy (i.e., > 4 days)
Myostatin (???) negates IGF-1Ea signaling after exercise (no myoblast differentiation occurs)
IGF-1Ea signals one proliferating myoblast to differentiate
Step 3: Synthesis of new myofibrillar proteins from 2 domains
- Differentiation and fusion of myoblast creates a new myonuclear domain
- Transcription of myofibrillar protein genes ensues following subsequent exericse
- Increase in ribosome content and translation of myofibrillar protein genes
- Semiconservative formation of new myofibrils (gray)

Figure 4.

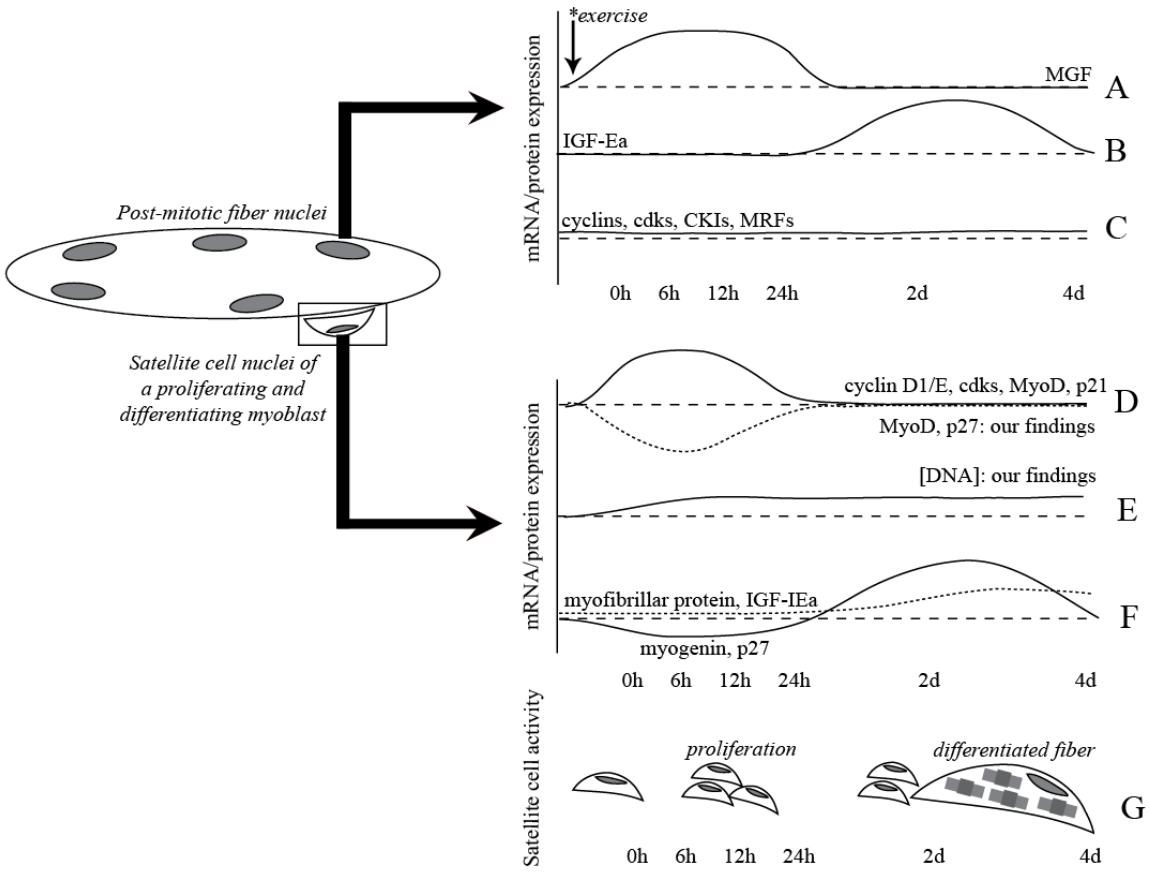

*exercise
MGF
A
IGF-Ea
B
cyclins, cdks, CKIs, MRFs
C
mRNA/protein expression
0h 6h 12h 24h 2d 4d
Post-mitotic fiber nuclei
Satellite cell nuclei of a proliferating and differentiating myoblast
cyclin D1/E, cdks, MyoD, p21
D
MyoD, p27: our findings
[DNA]: our findings
E
myofibrillar protein, IGF-IEa
F
myogenin, p27
mRNA/protein expression
0h 6h 12h 24h 2d 4d
Satellite cell activity
proliferation
differentiated fiber
G
0h 6h 12h 24h 2d 4d

Figure 5.

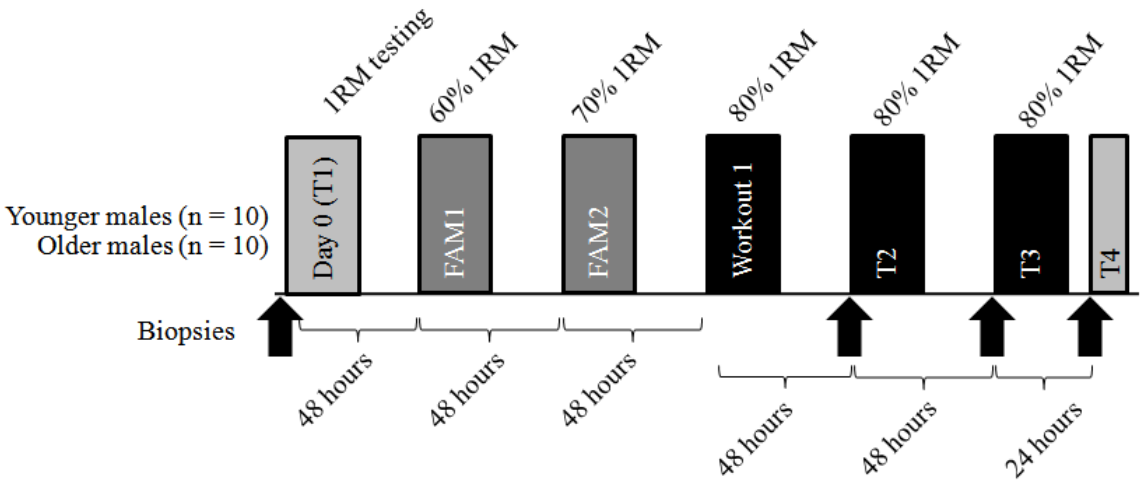
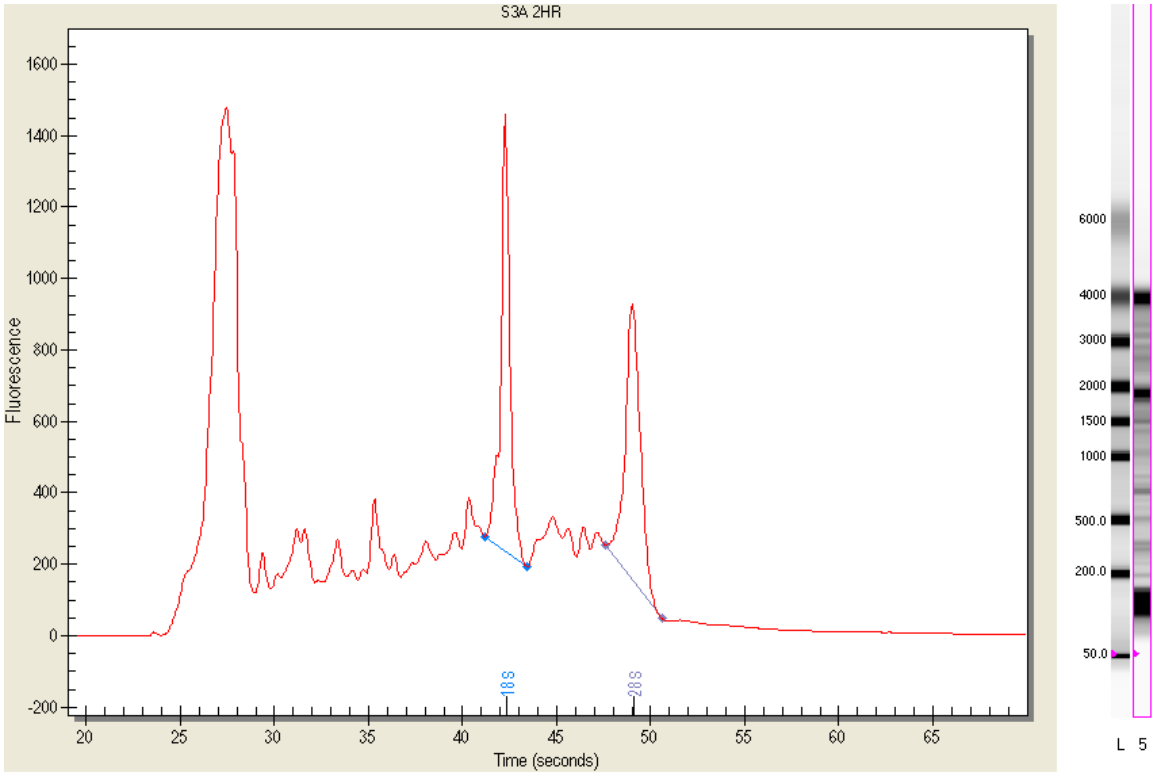

1RM testing
60% 1RM
70% 1RM
80% 1RM
80% 1RM
80% 1RM
Younger males (n = 10)
Older males (n = 10)
Day 0 (T1)
FAM1
FAM2
Workout 1
T2
T3
T4
Biopsies
48 hours
48 hours
48 hours
48 hours
48 hours
24 hours

Figure 6.



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

Figure 7.

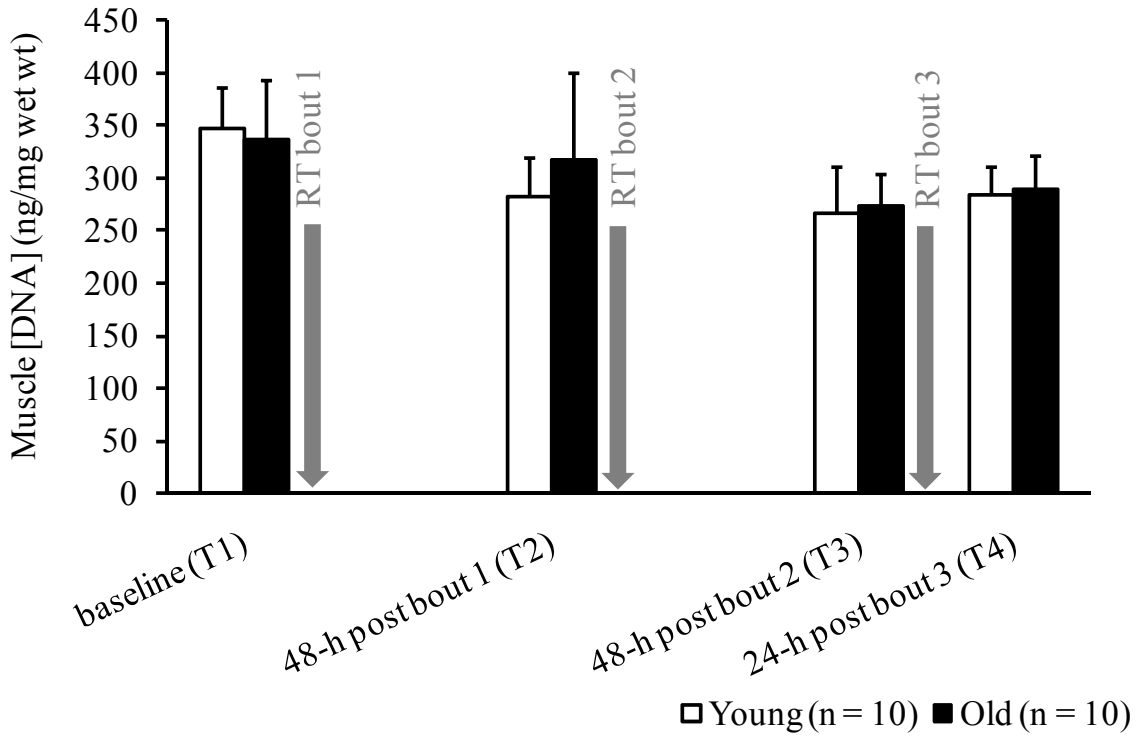

Muscle [DNA] (ng/mg wet wt)
450
400
350
300
250
200
150
100
50
0
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)

Figure 8.

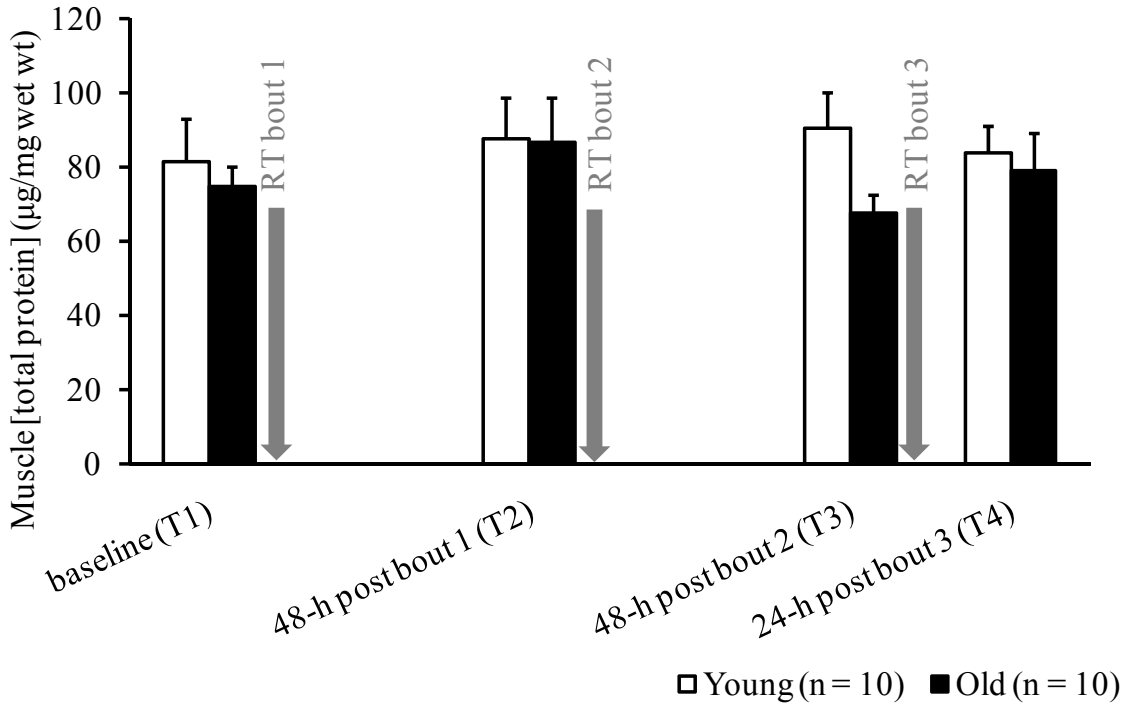

120
100
80
60
40
20
0
Muscle [total protein] (µg/mg wet wt)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)

Figure 9.

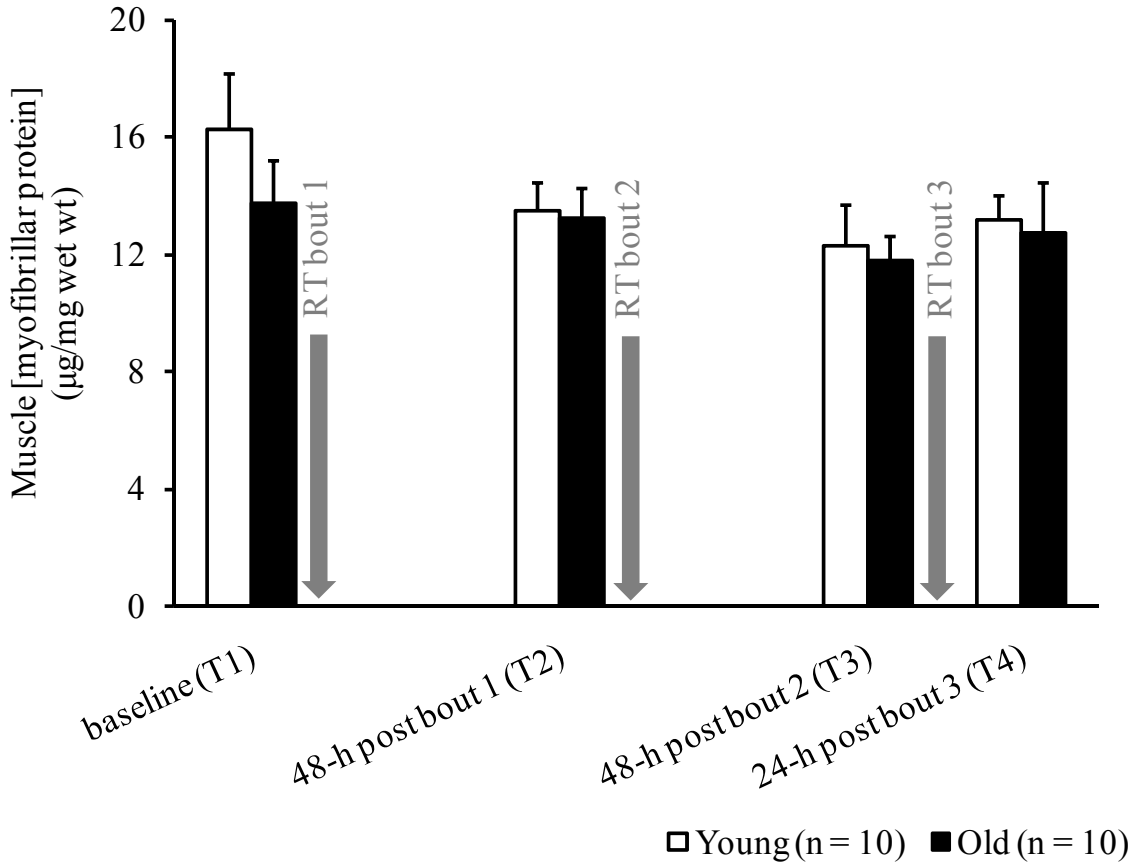

20
16
12
8
4
0
Muscle [myofibrillar protein] (µg/mg wet wt)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)

Figure 10.

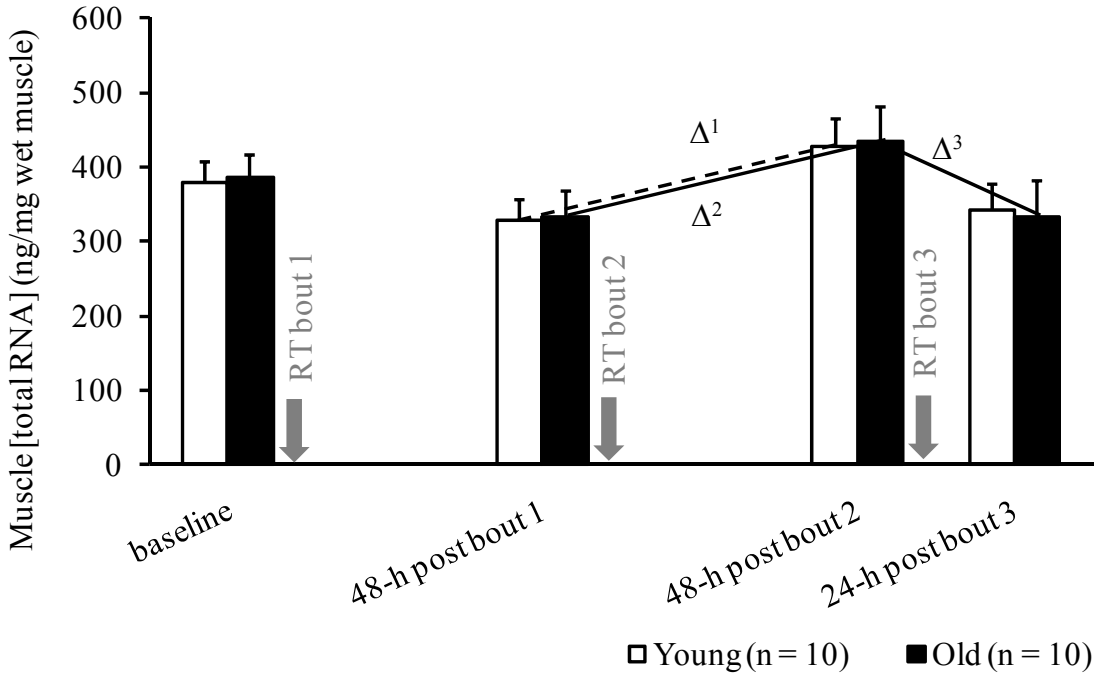

600
500
400
300
200
100
0
Muscle [total RNA] (ng/mg wet muscle)
RT bout 1
RT bout 2
RT bout 3
Δ1
Δ2
Δ3
baseline
48-h post bout 1
48-h post bout 2
24-h post bout 3
Young (n = 10)
Old (n = 10)

Figure 11.

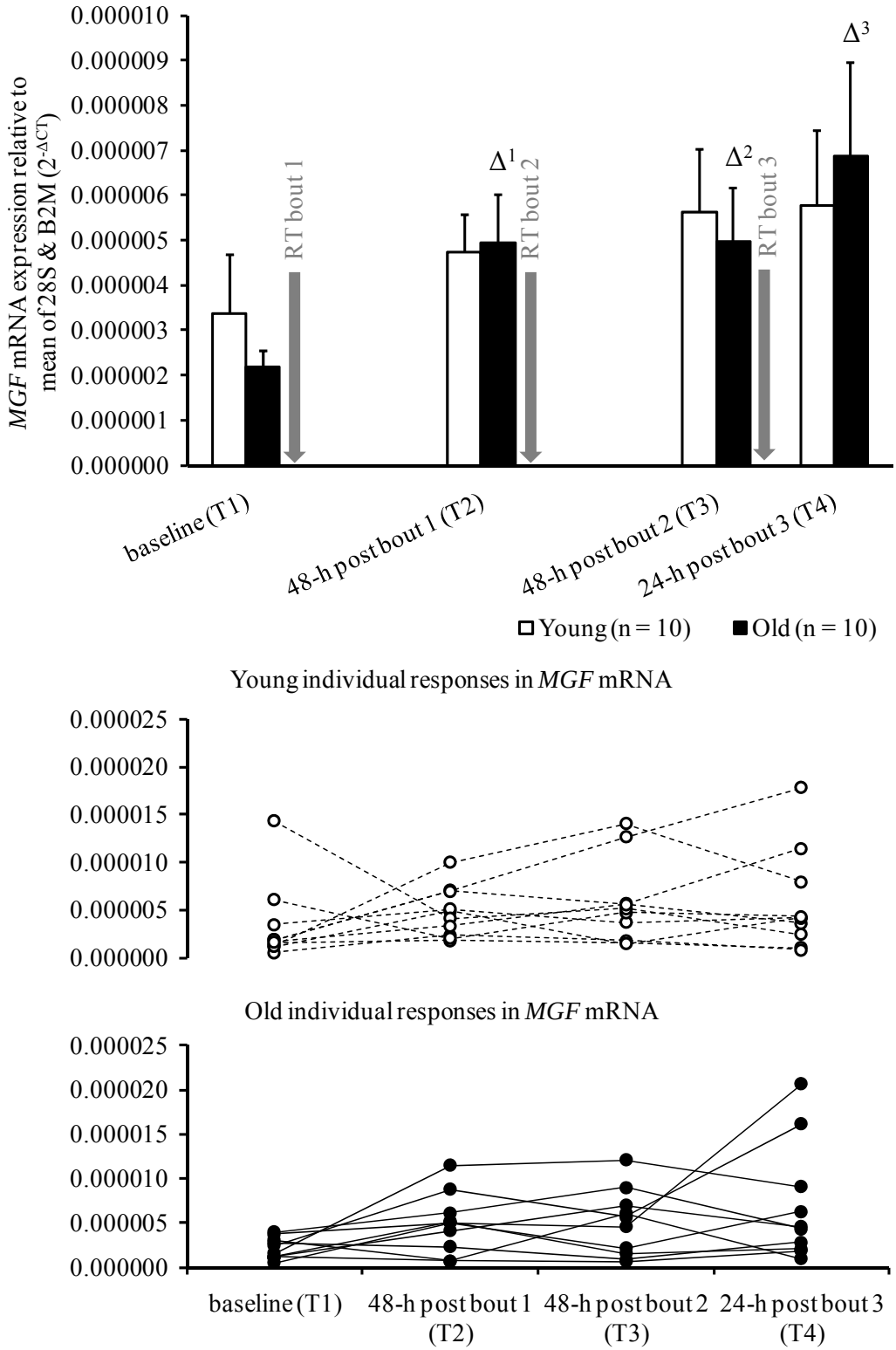

0.000010
0.000009
0.000008
0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0.000000
MGF mRNA expression relative to mean of 28S & B2M (2-ΔCT)
Δ1
Δ2
Δ3
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in MGF mRNA
0.000025
0.000020
0.000015
0.000010
0.000005
0.000000
Old individual responses in MGF mRNA
0.000025
0.000020
0.000015
0.000010
0.000005
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 12.

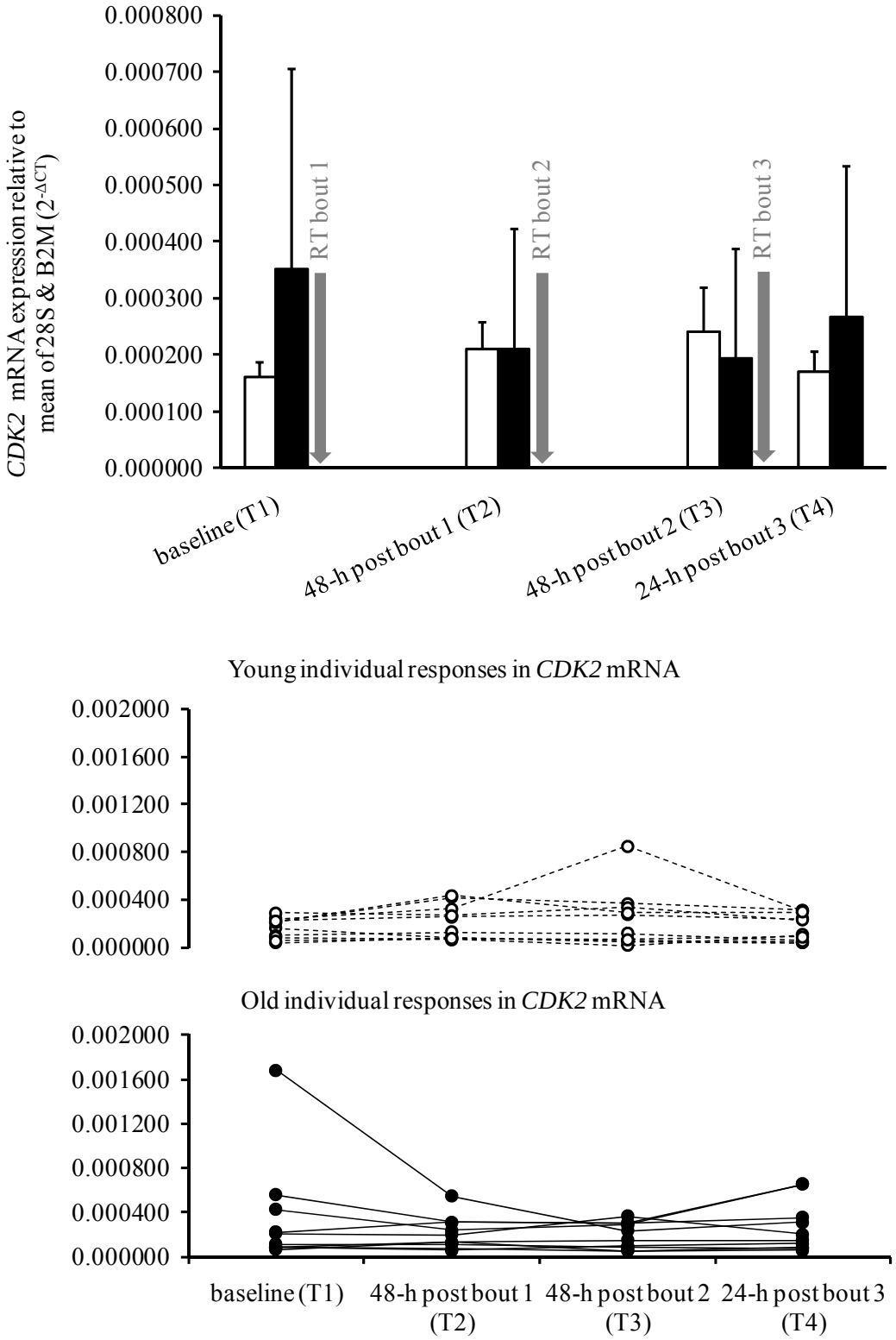

0.000800
0.000700
0.000600
0.000500
0.000400
0.000300
0.000200
0.000100
0.000000
CDK2 mRNA expression relative to mean of 28S & B2M (2-ΔCT)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young individual responses in CDK2 mRNA
0.002000
0.001600
0.001200
0.000800
0.000400
0.000000
Old individual responses in CDK2 mRNA
0.002000
0.001600
0.001200
0.000800
0.000400
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 13.

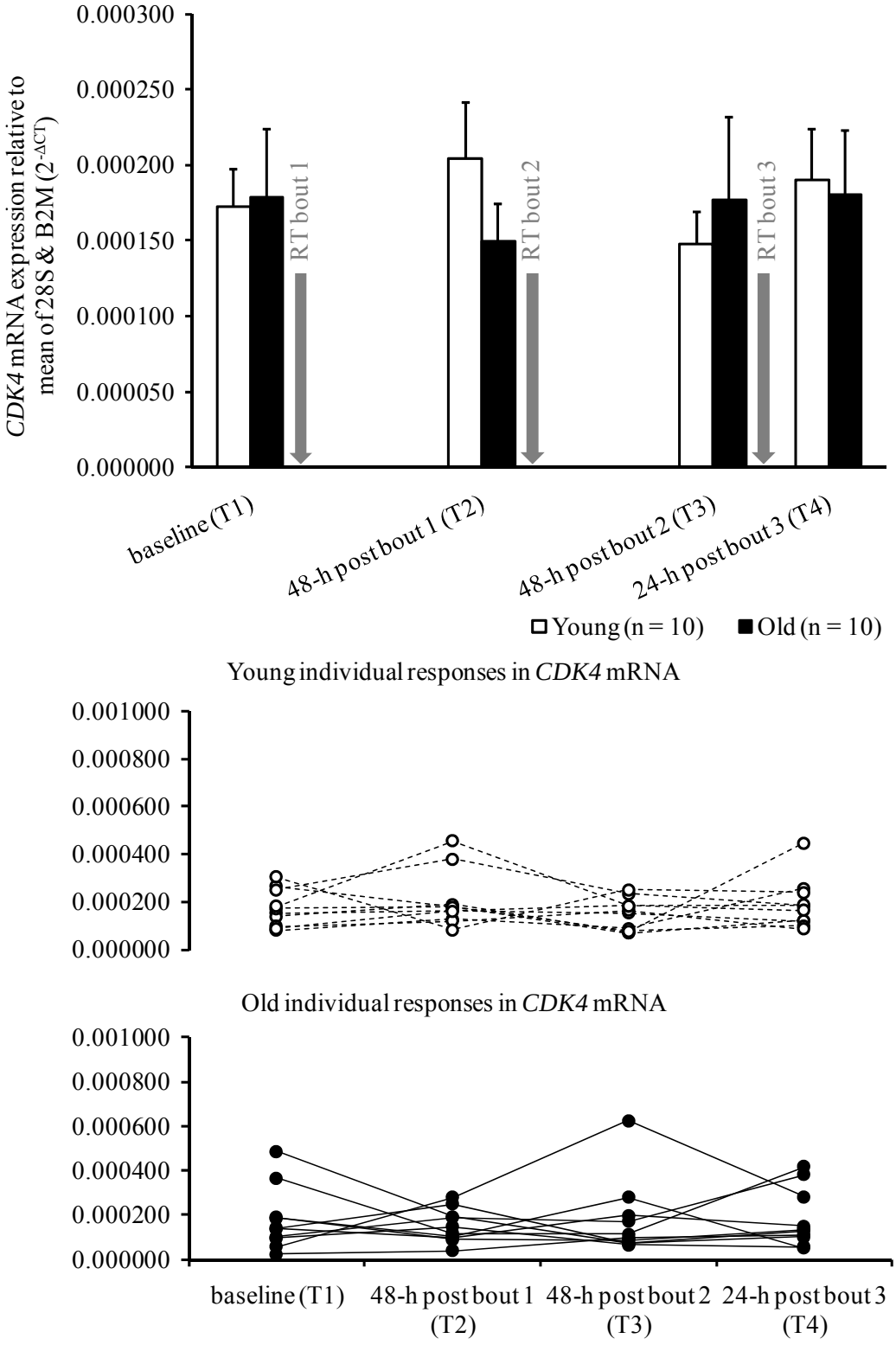

0.000300
0.000250
0.000200
0.000150
0.000100
0.000050
0.000000
CDK4 mRNA expression relative to mean of 28S & B2M (2-ΔCT)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in CDK4 mRNA
0.001000
0.000800
0.000600
0.000400
0.000200
0.000000
Old individual responses in CDK4 mRNA
0.001000
0.000800
0.000600
0.000400
0.000200
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 14.

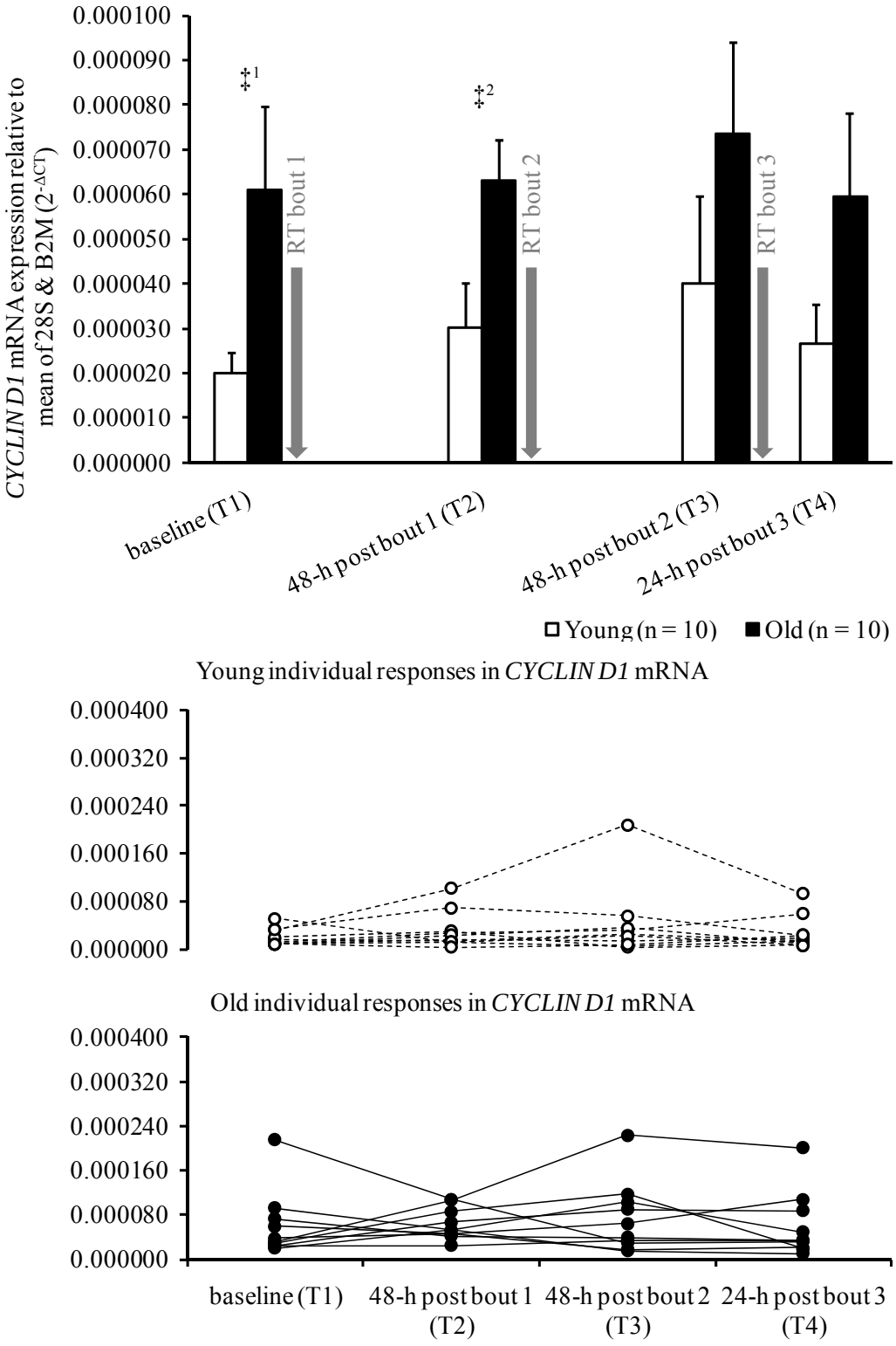

0.000100
0.000090
0.000080
0.000070
0.000060
0.000050
0.000040
0.000030
0.000020
0.000010
0.000000
CYCLIN D1 mRNA expression relative to mean of 28S & B2M (2-ΔCT)
‡1
‡2
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in CYCLIN D1 mRNA
0.000400
0.000320
0.000240
0.000160
0.000080
0.000000
Old individual responses in CYCLIN D1 mRNA
0.000400
0.000320
0.000240
0.000160
0.000080
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 15.

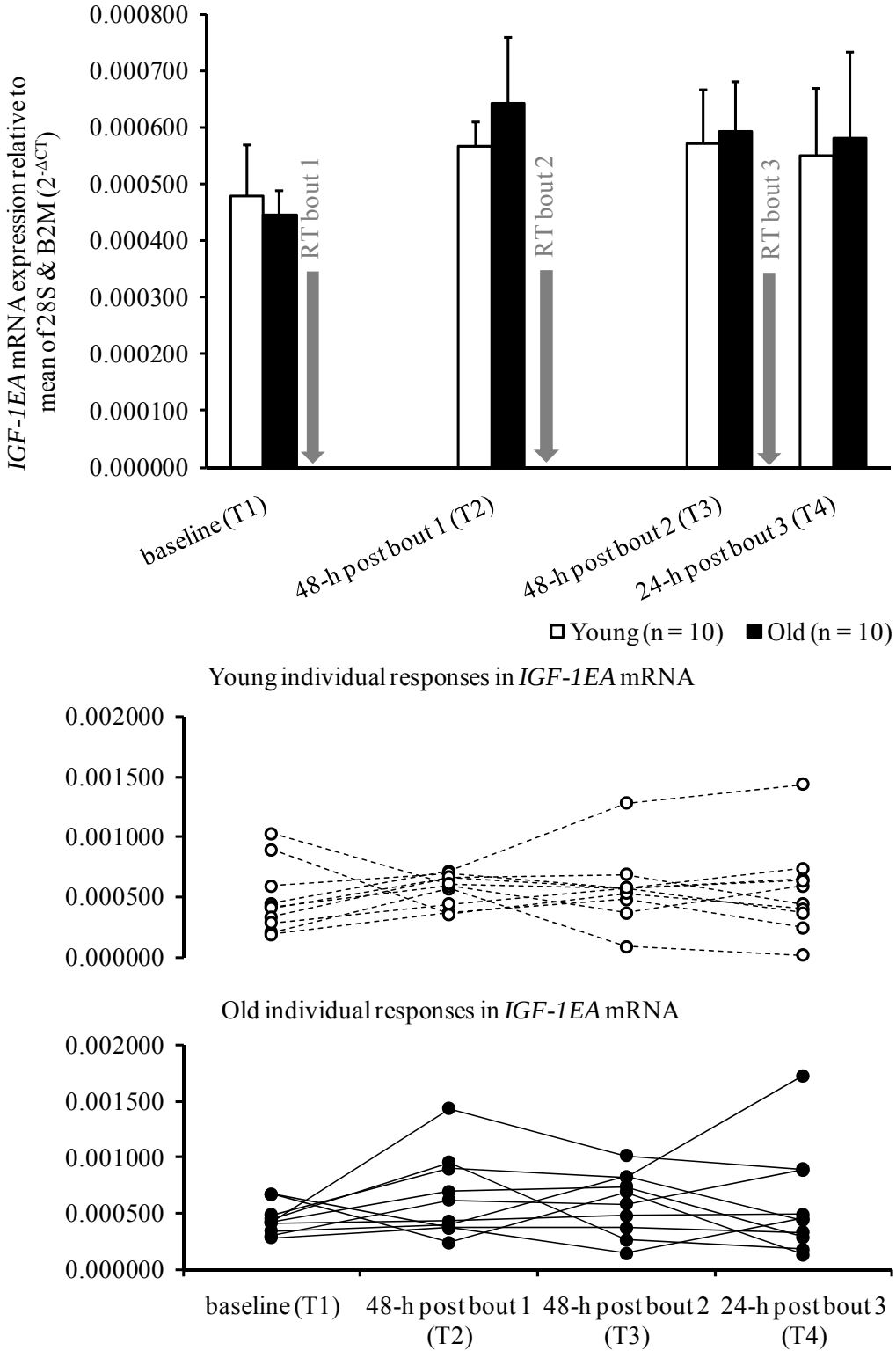

0.000800
0.000700
0.000600
0.000500
0.000400
0.000300
0.000200
0.000100
0.000000
IGF-1EA mRNA expression relative to mean of 28S & B2M (2-ΔCT)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in IGF-1EA mRNA
0.002000
0.001500
0.001000
0.000500
0.000000
Old individual responses in IGF-1EA mRNA
0.002000
0.001500
0.001000
0.000500
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 16.

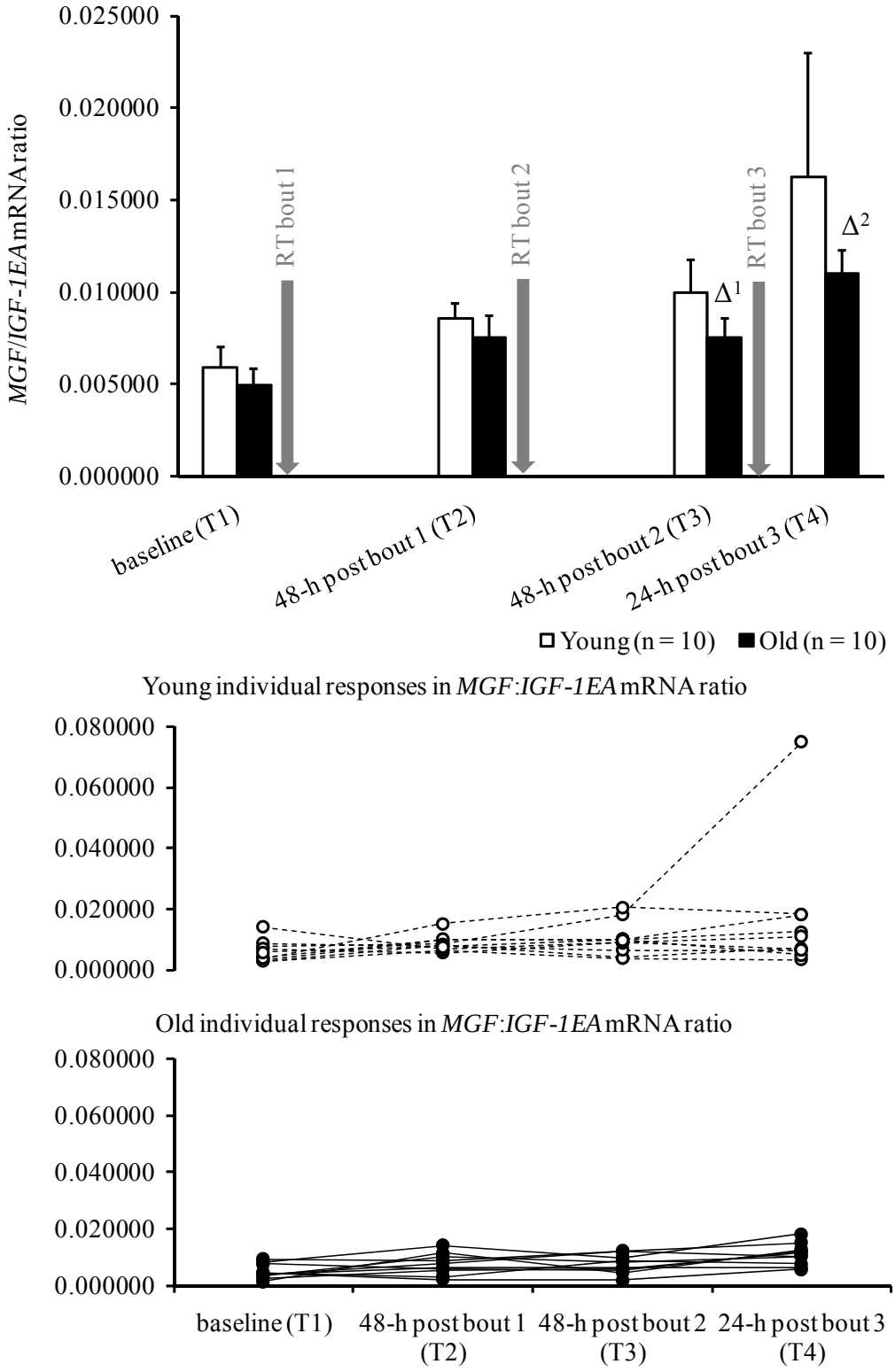

0.025000
0.020000
0.015000
0.010000
0.005000
0.000000
MGF/IGF-1EA mRNA ratio
RT bout 1
RT bout 2
RT bout 3
Δ1
Δ2
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in MGF:IGF-1EA mRNA ratio
0.080000
0.060000
0.040000
0.020000
0.000000
Old individual responses in MGF:IGF-1EA mRNA ratio
0.080000
0.060000
0.040000
0.020000
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 17.

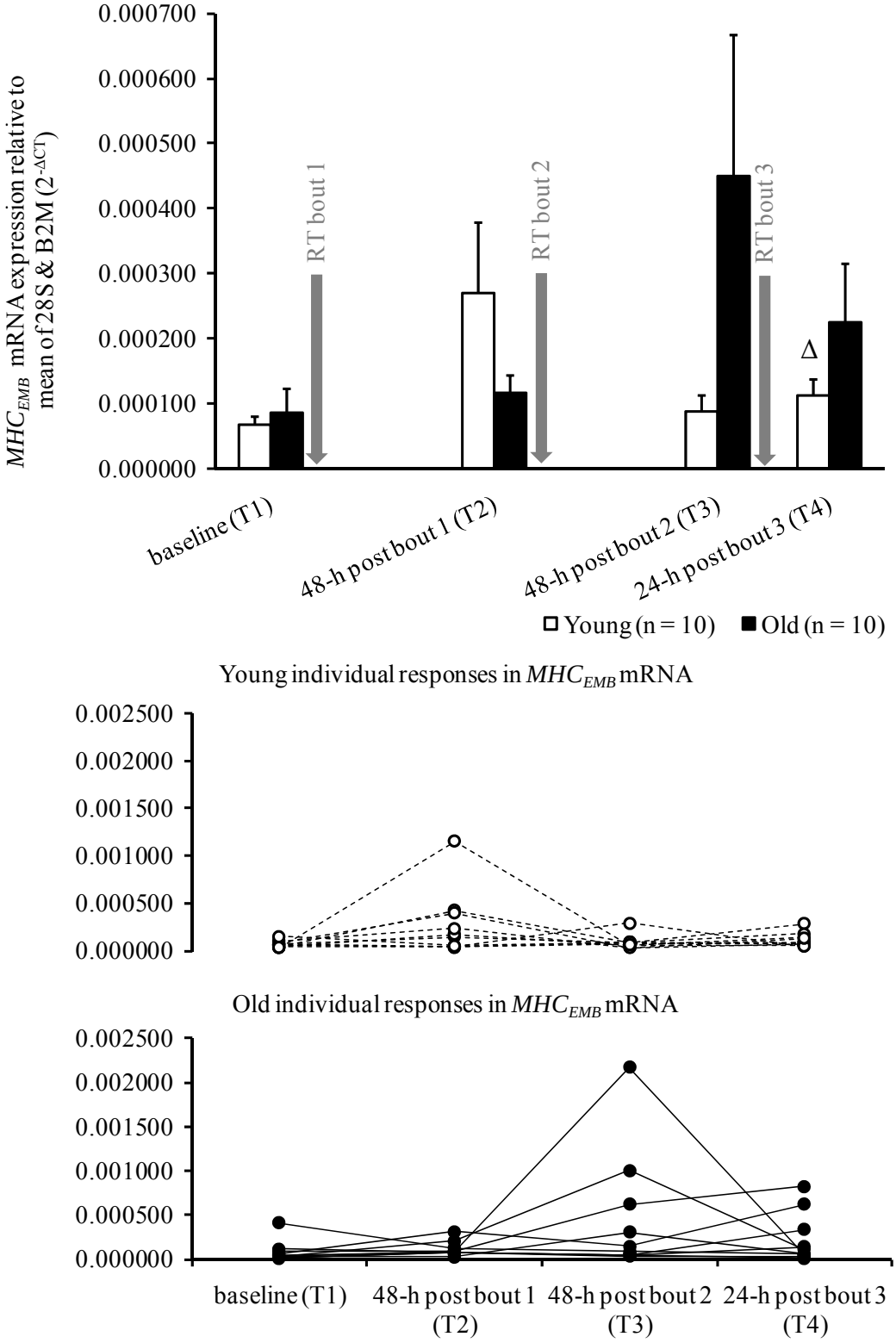

0.000700
0.000600
0.000500
0.000400
0.000300
0.000200
0.000100
0.000000
MHC_EMB mRNA expression relative to mean of 28S & B2M (2^-ΔCT)
RT bout 1
RT bout 2
RT bout 3
Δ
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in MHC_EMB mRNA
0.002500
0.002000
0.001500
0.001000
0.000500
0.000000
Old individual responses in MHC_EMB mRNA
0.002500
0.002000
0.001500
0.001000
0.000500
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 18.

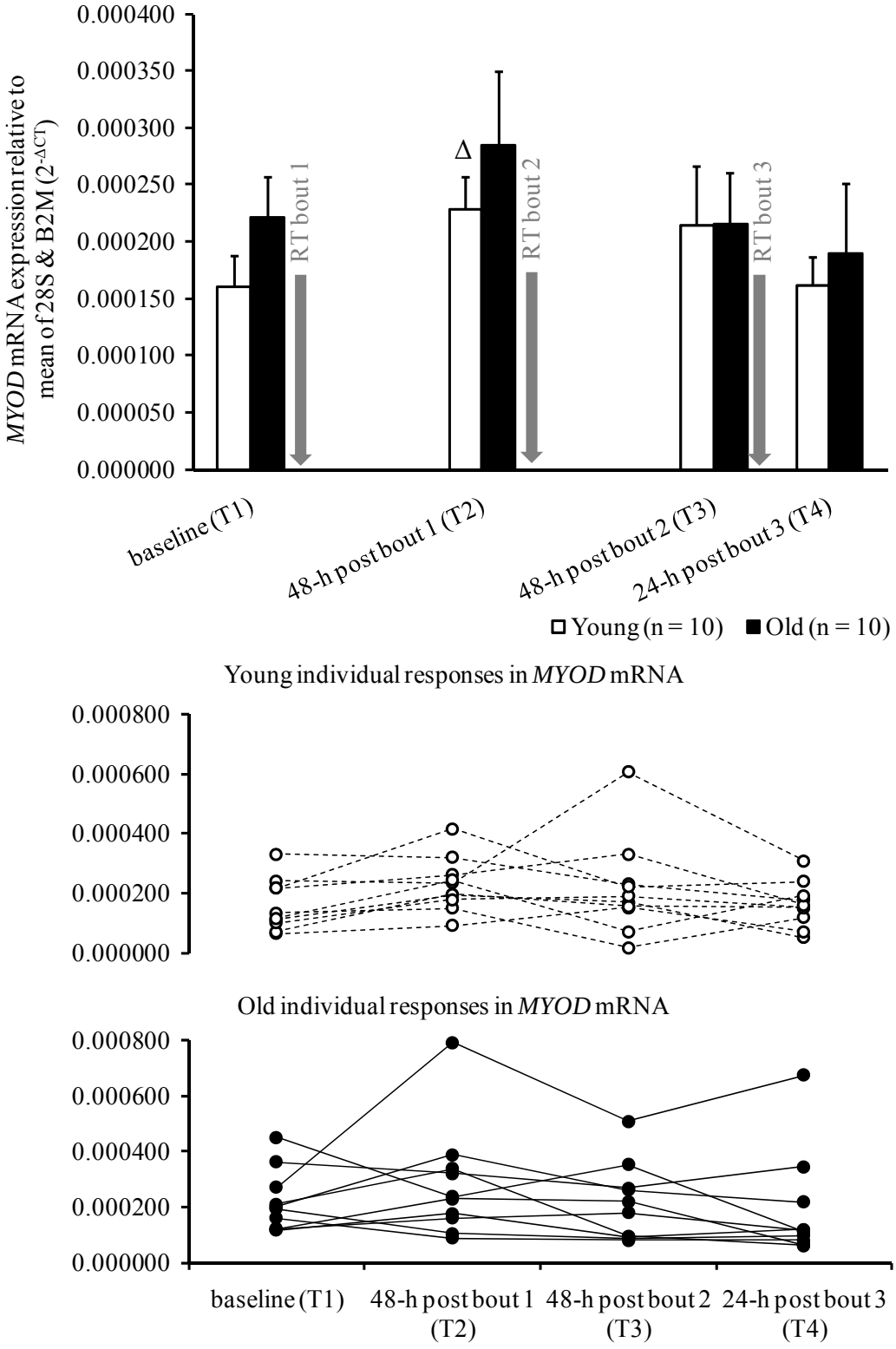

MYOD mRNA expression relative to mean of 28S & B2M (2-ΔCT)
0.000400
0.000350
0.000300
0.000250
0.000200
0.000150
0.000100
0.000050
0.000000
Δ
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in MYOD mRNA
0.000800
0.000600
0.000400
0.000200
0.000000
Old individual responses in MYOD mRNA
0.000800
0.000600
0.000400
0.000200
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 19.

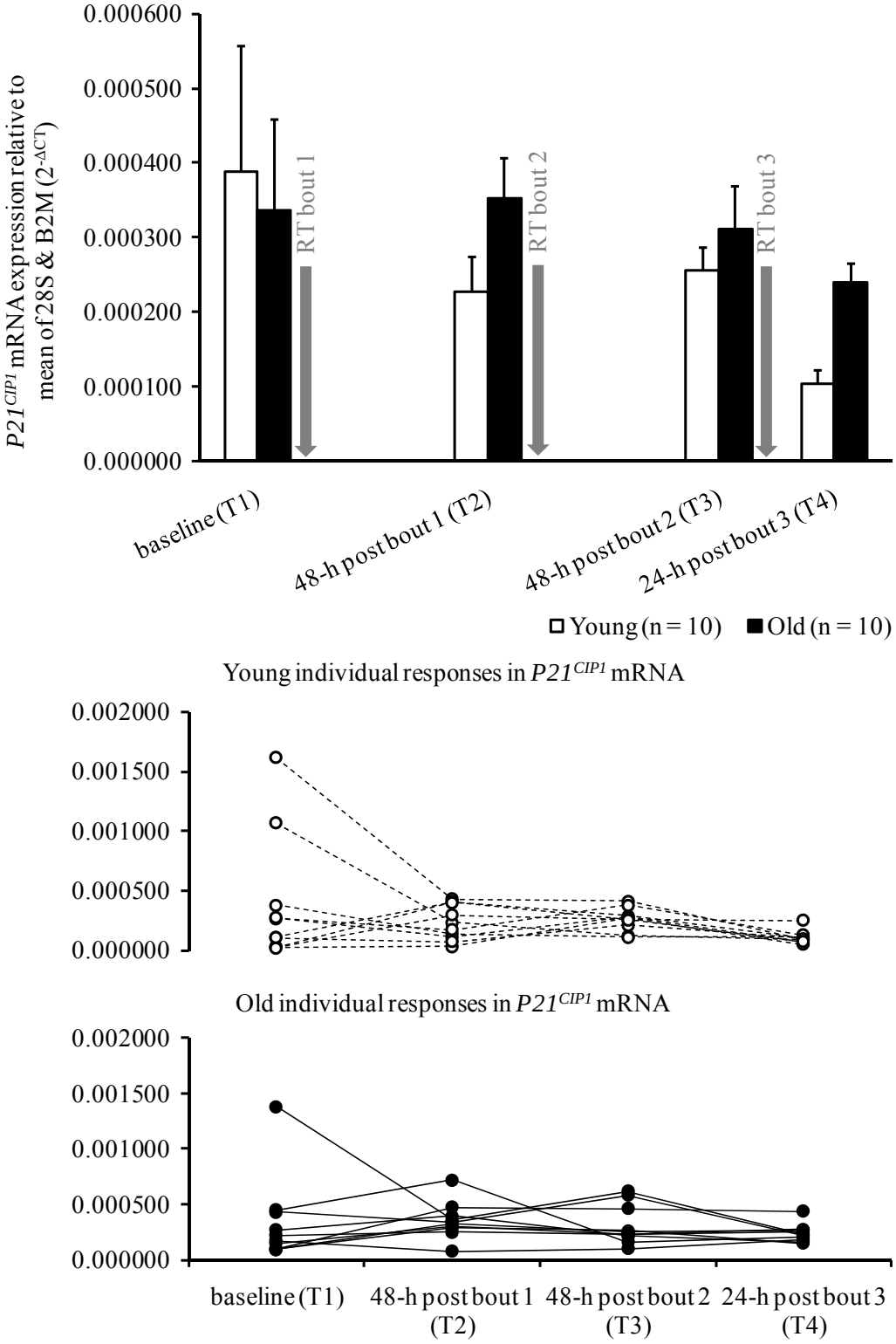

0.000600
0.000500
0.000400
0.000300
0.000200
0.000100
0.000000
P21CIP1 mRNA expression relative to mean of 28S & B2M (2-ΔCT)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in P21CIP1 mRNA
0.002000
0.001500
0.001000
0.000500
0.000000
Old individual responses in P21CIP1 mRNA
0.002000
0.001500
0.001000
0.000500
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 20.

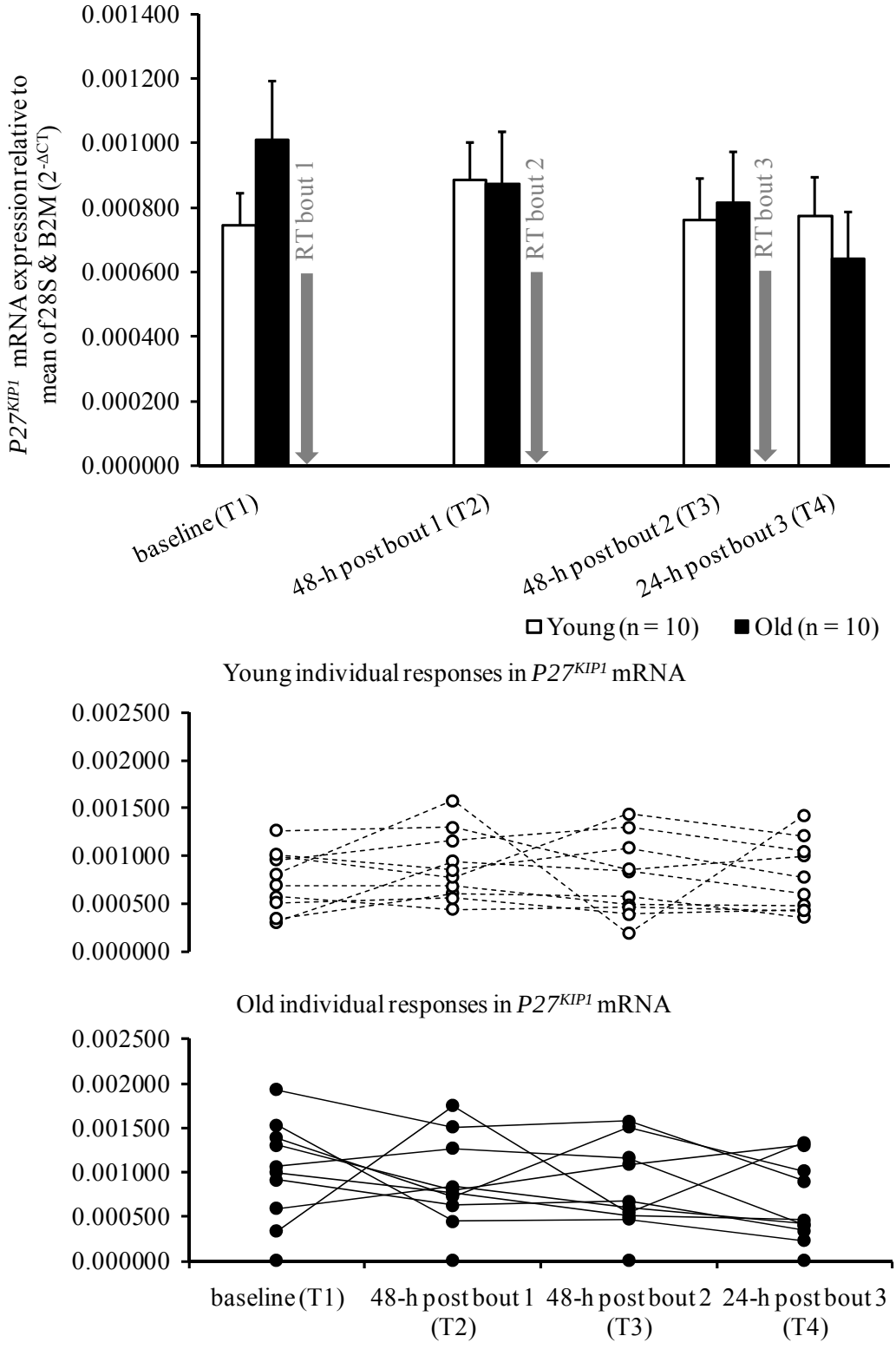
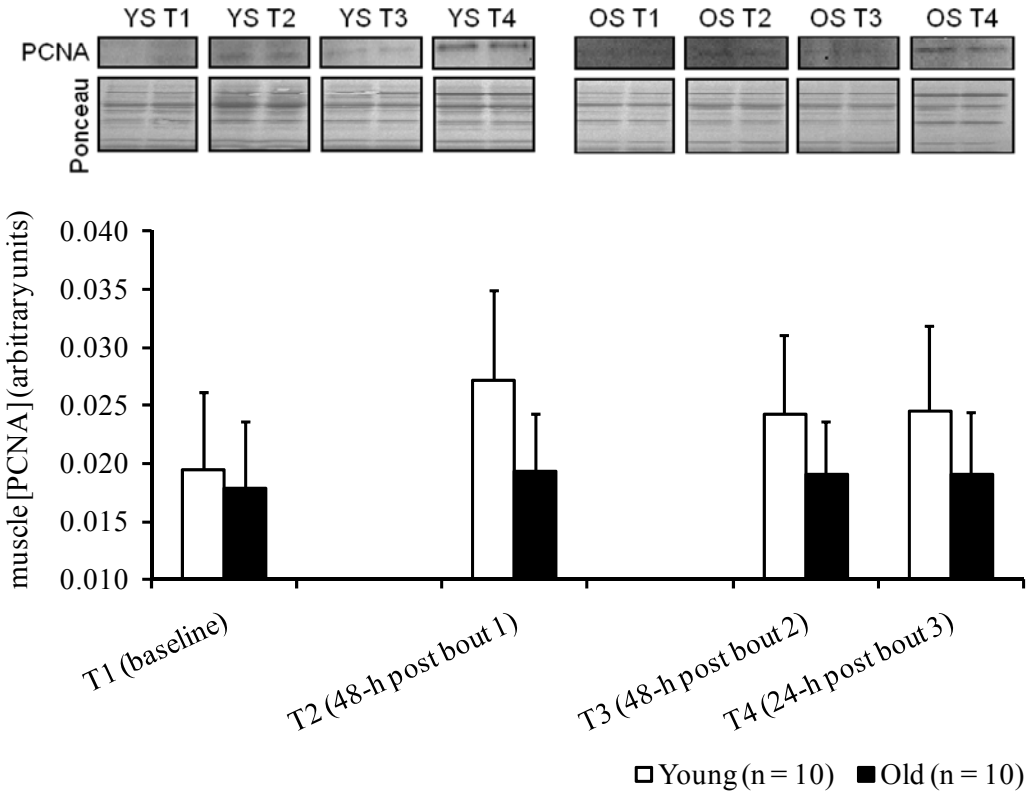

0.001400
0.001200
0.001000
0.000800
0.000600
0.000400
0.000200
0.000000
P27KIP1 mRNA expression relative to mean of 28S & B2M (2-ΔCT)
RT bout 1
RT bout 2
RT bout 3
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)
Young (n = 10)
Old (n = 10)
Young individual responses in P27KIP1 mRNA
0.002500
0.002000
0.001500
0.001000
0.000500
0.000000
Old individual responses in P27KIP1 mRNA
0.002500
0.002000
0.001500
0.001000
0.000500
0.000000
baseline (T1)
48-h post bout 1 (T2)
48-h post bout 2 (T3)
24-h post bout 3 (T4)

Figure 21.


YS T1
YS T2
YS T3
YS T4
OS T1
OS T2
OS T3
OS T4
PCNA
Ponceau
muscle [PCNA] (arbitrary units)
0.040
0.035
0.030
0.025
0.020
0.015
0.010
T1 (baseline)
T2 (48-h post bout 1)
T3 (48-h post bout 2)
T4 (24-h post bout 3)
Young (n = 10)
Old (n = 10)

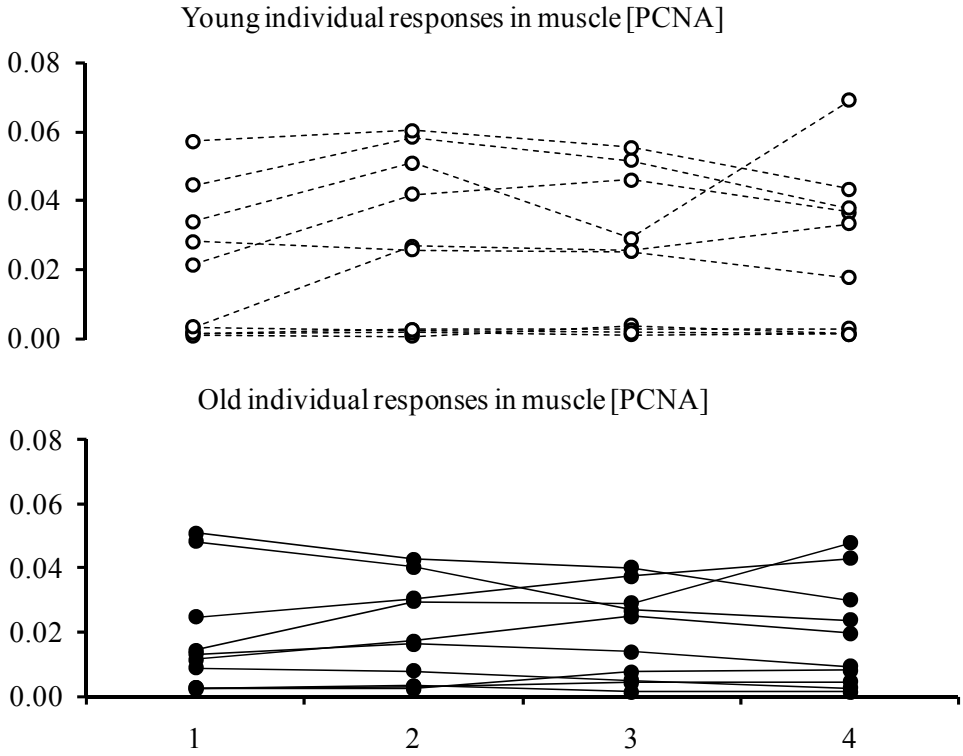

Young individual responses in muscle [PCNA]
0.08
0.06
0.04
0.02
0.00
Old individual responses in muscle [PCNA]
0.08
0.06
0.04
0.02
0.00
1
2
3
4

Figure 22.

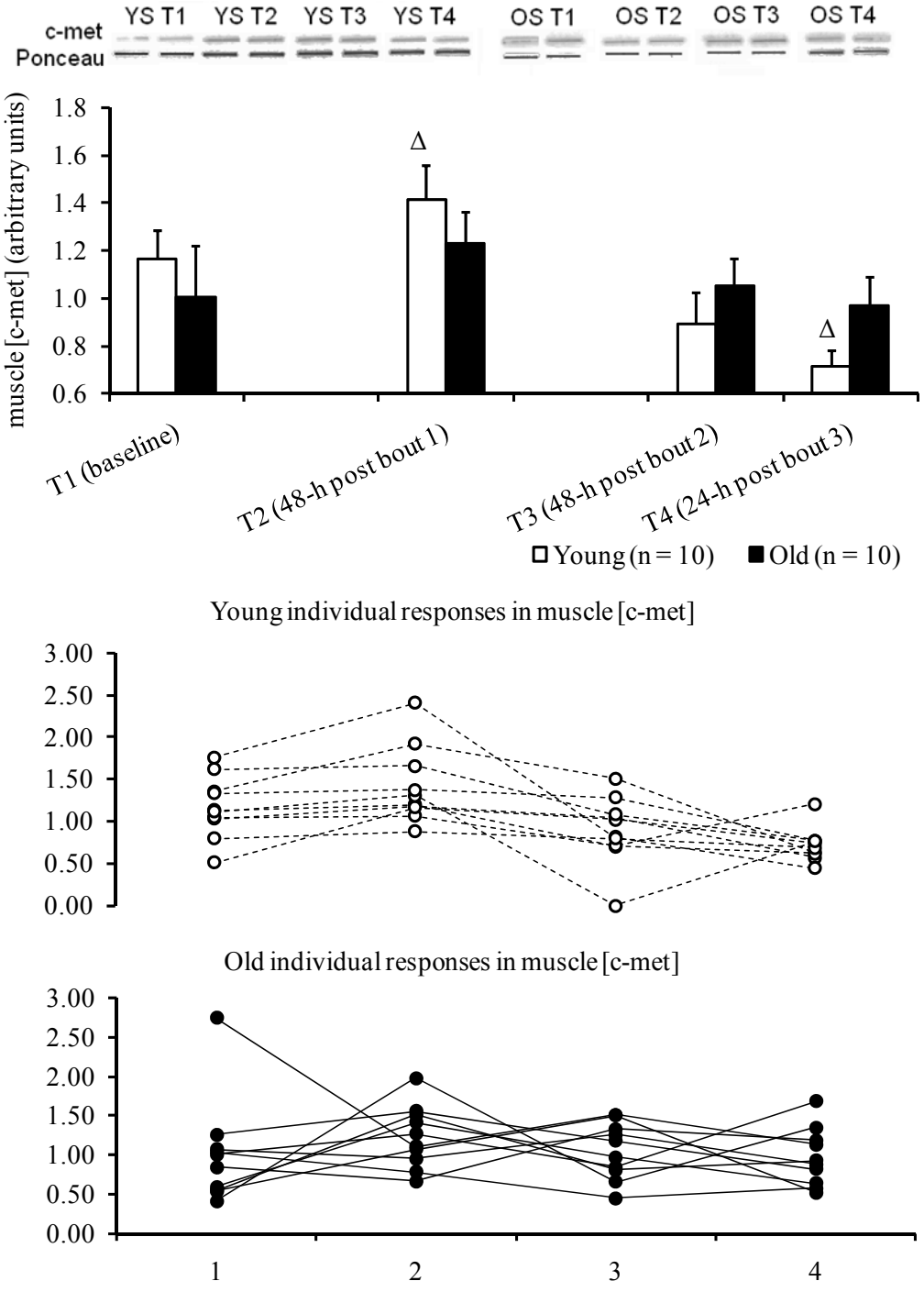
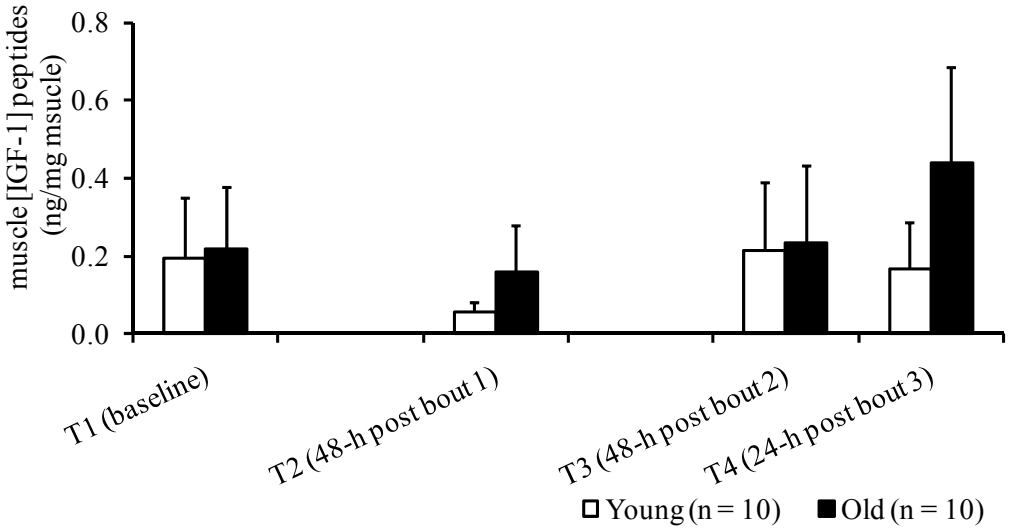
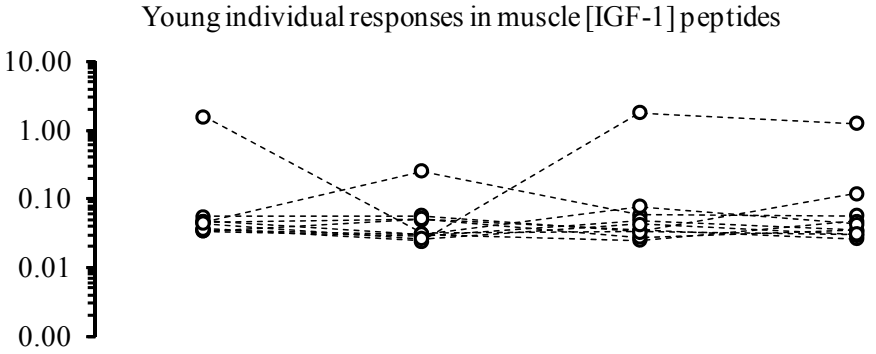
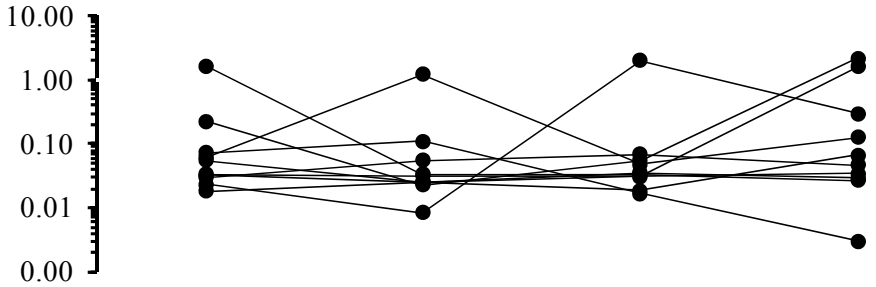

YS T1
YS T2
YS T3
YS T4
OS T1
OS T2
OS T3
OS T4
c-met
Ponceau
muscle [c-met] (arbitrary units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
Δ
Δ
T1 (baseline)
T2 (48-h post bout 1)
T3 (48-h post bout 2)
T4 (24-h post bout 3)
Young (n = 10)
Old (n = 10)
Young individual responses in muscle [c-met]
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Old individual responses in muscle [c-met]
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4

Figure 23.


muscle [IGF-1] peptides (ng/mg msucle)
0.8
0.6
0.4
0.2
0.0
T1 (baseline)
T2 (48-h post bout 1)
T3 (48-h post bout 2)
T4 (24-h post bout 3)
Young (n = 10)
Old (n = 10)


Young individual responses in muscle [IGF-1] peptides
10.00
1.00
0.10
0.01
0.00


Old individual responses in muscle [IGF-1] peptides
10.00
1.00
0.10
0.01
0.00

APPENDIX B

Table 1. Selected research demonstrating that acute and chronic exercise increases satellite cell activity.

Authors (PI when not listed as first author) – year (reference)	Study methods	Major findings
	Acute exercise settings	
Darr and Shultz – 1987 [132]	*Species* – rats *Exercise* – 1 bout of downhill treadmill running	Exercise increased ^{3}H-thymidine incorporation by 250% by 72 h post-exercise compared to non-exercise controls ($p < 0.05$)
Jacobs et al. – 1995 [133]	*Species* – rats *Exercise* – 1 bout of downhill treadmill running	Exercise consistently increased SC # starting from 2 d to 2 wk following exercise ($p < 0.05$)
Smith et al. – 2001 [134]	*Species* – rats *Exercise* – 1 to 7 days of downhill treadmill running	1. 1 to 7 days of running increased SC # in soleus and quadriceps muscles but not the TA muscle ($p < 0.05$) 2. 2 days of running increased number of SCs compared to 1 d of running in all muscles ($p < 0.05$) 3. Myonuclear # per fiber did not increase following 1, 2, 4, or 7 days of running
Crameri et al. – 2004 [65]	*Species* – younger (25 y) human males *Exercise* – 1 bout (210 reps) of 3 different eccentric leg extensor resistance exercises	SC # increased 146-192% at 2-8 d following exercise ($p < 0.05$)

Dreyer et al. – 2006 [16]♦♦	*Species* – younger (23-35 y) and older (60-75 y) human males *Exercise* – 1 bout (92 reps) of eccentric leg extensor resistance exercise	24-h post SC # per fiber increased to a greater extent among the young men (141%; $p < 0.001$) than older men (51%; $p = 0.002$) from pre-exercise levels
O'Reilly et al. (Parise) – 2008 [55]	*Species* – younger (21 y) human males *Exercise* – 1 bout (300 reps) eccentric leg extensor resistance exercises	1. After the exercise bout, SC # increased 138% (1 d), 147% (3 d), and 118% (5 d) ($p < 0.05$) 2. Myonuclear # per mature myofiber was not different through 5 d post-exercise
Prolonged exercise settings		
James and Cabric – 1981 [135]	*Species* – rats *Exercise* – 2 daily bouts of swimming (30 min per d) for 35 days	1. Mean CSA of muscle fibers unaltered 2. 30% increase in myonuclear # per unit volume of fibers ($p < 0.05$)
Umnova and Seene – 1991 [136]	*Species* – rats *Exercise* – 6 weeks of treadmill running	10% increase in SC # of trained animals versus 3% in non-exercised controls ($p < 0.05$)

Kadi and Thornell – 2000 [39]$^{\delta}$	*Species* – middle-aged (38 y) female humans *Exercise* – 3 weekly bouts of full body training for 10 weeks	1. increases trapezius muscle SC # by 46% ($p < 0.05$), myonuclear # by 70% ($p < 0.05$) 2. rise in SC # maintained for a period up to 60 days post-training during detraining 3. SC # return to pre-training levels at 90 days signifying that physical inactivity may induce apoptotic mechanisms to reduce SC #
Roth et al. – 2001 [118]$^{\blacklozenge\blacklozenge}$	*Species* – younger (20-30 y) and older (65-75 y) males and female humans *Exercise* – 3 weekly bouts of leg extensions (55 reps per bout) for 9 weeks	1. All subjects experienced an increase in SC # following training ($p < 0.05$) 2. Older women exhibited largest increase in SC # following training
Charifi (Kadi) et al. – 2003 [62]	*Species* – older males (70-80 y); no younger cohort was examined *Exercise* – 4 weekly bouts of cycling (45 min per d) for 14 weeks	1. No increase in myonuclei per fiber 2. 22% increase in SC # per fiber ($p < 0.05$)
Petrella et al. (Bamman) – 2006 [15]$^{\blacklozenge\blacklozenge}$	*Species* – younger (20-25 y) and older (60-75 y) male and female humans *Exercise* – 3 weekly bouts of RT of leg extensors (~90 reps) for 16 weeks	1. Myofiber hypertrophy occurred in all groups, but was twofold greater in YM vs. others OM, YW, OW ($p < 0.05$) 2. Only YM increased NCAM+ cells per 100 myofibers (49%) and myonuclei per fiber (19%) ($p < 0.05$)

Verney (Kadi) et al. – 2008 [137]	*Species* – older males (70-80 y); no younger cohort was examined *Exercise* – 3 weekly bouts of RT of deltoids for 14 weeks; 3 weekly bouts of cycling bouts per week of VLat for 14 weeks	1. 38% increase in SC # per fiber in ET and RT muscles ($p < 0.05$) 2. Increases in SC # occurred more in type II fibers 3. No change in myonuclei # per fiber in ET or RT muscles
Petrella et al. (Bamman) – 2008 [14]$^{\delta\delta}$	*Species* – 66 humans; (Xtr, n = 17), modest (Mod, n = 32), or nonresponders (Non, n = 17) based on myofiber hypertrophy, which averaged 58, 28, and 0%, respectively *Exercise* – 3 weekly bouts of knee extensor resistance training (~90 reps per session) for 16 weeks	1. At baseline, myofiber size did not differ among clusters; however, SC # was greater in Xtr ($p < 0.01$) than both Mod and Non 2. SC # increased following training in Xtr only (117%, $p < 0.001$), and Xtr had more myonuclei per fiber than Non (23%, $p < 0.05$) and tended to have more than Mod (19%, $p = 0.056$)
Verdijk et al. – 2009 [12]	*Species* – older (72 y) human males *Exercise* – 3 weekly bouts of leg extensor resistance training (64-120 reps per session) for 12 weeks	1. Leg strength increased 25%-30% after training ($p < 0.001$) 2. Leg lean mass and quadriceps cross-sectional area increased 6%-9% ($p < 0.001$) 3. Type II fiber CSA increased 28% ($p < 0.01$) 4. Type II muscle fiber SC # increased 75% ($p < 0.001$); no changes were observed in the Type I muscle fibers

Mackey et al. (Kadi) – 2009 [63]	*Species* – younger (21 y) human males *Exercise* – 3 weekly bouts of high intensity (80 reps at 70% 1RM) versus low intensity leg extensions (360 reps at 15% 1RM) for 12 weeks	2.4% and 1.4% increase in active SCs (assessed by Ki67+ cells; total SC not assessed) in the low-intensity and high-intensity groups, respectively, 48 h following the last training bout ($p < 0.05$)

Unless specified, resistance training intensities in the human studies were ~70-80% 1RM (or 8-12 RM).
Abbreviations and symbols – 1RM: one repetition maximum; CSA: cross-sectional area; ET: endurance training; OF; old female; OM: old male; PI: principal investigator; RT: resistance training; SC: satellite cell; VLat: vastus lateralis; YF: young female; YM: young male; ♦♦: study compared the satellite cell activity of younger versus older subjects (1 acute and 2 prolonged settings); δ: important point of contention whereby Kadi and Thornell [39] suggest that the rapid exercise-induced increase in satellite cell number may be reduced via apoptosis during periods of inactivity; δδ: important point of contention whereby Petrella et al. [14] determined that hypertrophic potential appears to be reliant upon the pre-existing number of satellite cells

Table 2. Primer sequences used to probe genes of interest expressed in skeletal muscle samples

Gene	Primer sequence (forward and reverse)	GenBank accession #
CDK2	5'- GGC TGG GAG ACT GAA GAC -3' 5'- GTA GAA GGG ACA AAC AAG GG -3'	NM_052827
CDK4	5'- GGA GTG AGC AAT GGA GTG -3' 5'- GGA AGG AGA AGG AGA AGC -3'	NM_000075
CYCLIN D1	5'- TTG GTT ACA GTA GCG TAG -3' 5'- TTA TAG TAG CGT ATC GTA GG -3'	NM_053056
IGF-1EA	5'- GAC ATG CCC AAG ACC CAG AAG GA -3' 5'- CGG TGG CAT GTC ACT CTT CAC TC -3'	X57025
MGF	5'- CGA AGT CTC AGA GAA GGA AAG G -3' 5'- ACA GGT AAC TCG TGC AGA GC -3'	U40870
MHC_{EMB}	5'- CAA GAA TGA GGA AAT TCA GAG G -3' 5'- CTT GCT CCT GGA AAG TTG G -3'	X15696
MYOD	5'- CAC AAC GGA CGA CTT CTA TG -3' 5'- TGC TCT TCG GGT TTC AGG -3'	NM_002478
$P21^{CIP1}$	5'- CAG CAT GAC AGA TTT CTA CC -3' 5'- GGA ATC AGA GTC AAA CAC AC -3'	L25610
$p27^{KIP1}$	5'- CAG GAG AGC CAG GAT GTC-3' 5'- TAG AAG AAT CGT CGG TTG C-3'	NM_004064

Abbreviations – cdk: cyclin dependent kinase, IGF-1Ea: insulin-like growth factor-1Ea; MGF: mechano growth factor; MHC_{emb}: embryonic myosin heavy chain; MyoD: myogenic differentiation 1; $p21^{Cip1}$: p21 cdk-interacting protein 1; $p27^{Kip1}$: p27 cdk-inhibitor 1B

Table 3. Normality distribution tests for all dependent variables

Shapiro-Wilk statistic p-values for each dependent variable (H_0: data is normally distributed)				
Dependent Variable	*T1 p-value*	*T2 p-value*	*T3 p-value*	*T4 p-value*
Muscle [total RNA]	0.67	0.95	0.17	0.51
Muscle [DNA]	0.22	0.36	0.61	0.17
Muscle [total protein]*	0.029	0.014	0.19	0.34
Muscle [myofibrillar protein]*	0.40	0.30	0.27	0.022
MGF mRNA*	< 0.001	0.36	0.07	0.007
CDK2 mRNA*	< 0.001	0.041	0.003	0.004
CDK4 mRNA*	0.09	0.38	0.002	0.43
CYCLIN D1 mRNA*	< 0.001	0.38	0.003	0.008
IGF-1EA mRNA*	0.035	0.059	0.85	0.025
MGF:IGF-1EA mRNA*	0.022	0.95	0.72	0.23
MHC_{EMB} mRNA*	0.44	0.014	< 0.001	0.001
MYOD mRNA*	0.036	0.008	0.12	< 0.001
$P21^{CIP1}$ mRNA	0.06	0.40	0.73	0.18
$P27^{KIP1}$ mRNA	1.00	0.69	0.52	0.54
Muscle [PCNA]*	0.004	0.021	0.042	0.025
Muscle [c-met]*	0.011	0.37	0.39	0.031
Muscle [IGF-1]*	< 0.001	< 0.001	< 0.001	< 0.001

Symbols – *: indicates that data at one or multiple time points from T1-T4 was not normally distributed and nonparametric statistics were used to compared between- and within-group means

Appendix C

FIGURE LEGENDS

Figure 1. Summary of Mauro's hypotheses on the origin of satellite cells (from reference [35]):

A) Mauro comments upon the possibility that a myonucleus of a damaged myofiber may dedifferentiate an pinch off to form a satellite cell which is then able to re-contribute itself to the damaged myofiber; a hypothesis which Mauro himself is uncharacteristic.
B) Mauro hypothesized that the satellite cells may arise from residual cells that did not fuse during embryonic and post-natal myogenesis; a hypothesis which was later confirmed using time-lapse microscopy [40].
C) Mauro also commented upon the possibility of wandering stem cells infiltrating muscle tissue to contribute to the satellite cell population; a hypothesis which may also hold true due to the presence of multipotent stem cells in the muscle bed [42].

Figure 2. The behavioral comparison of "conventional" satellite cells versus multipotent satellite stem cells.

A) The depiction of two phenotypically distinct satellite cell populations as described by Zammit et al. [42] in which the majority group of resident satellite cells rapidly proliferates following an acute mitogenic (i.e., exercise) stimulus whereas the minority group of satellite stem cells undergoes prolonged proliferation periods.
B) The depiction of these two cell lines proliferating following a series of mitogenic events followed by the eventual differentiation and fusion of a single myoblast to the pre-existing fiber. Note that satellite stem cells (~10%; possibly mesenchymal and/or hematopoietic stem cells) are multipotent in that they can also differentiate into a non-myogenic cellular species under other circumstances (i.e., vascular cell species via vascular growth factor signaling).

Figure 3. The manner in which myonuclear domains plausibly regulate satellite cell activity. Mechanistically, this process may occur by virtue of the myonuclear domain expressing a negative growth factor (myostatin???) signal which quells satellite cell activation, proliferation, and differentiation. The following steps describe how an early-phase increase in hypertrophy expands the myonuclear domain which obviates the need for myonuclear addition:

Step 1 (within 1-2 days ???): The splitting of myofibrils occurs due to intermittent, forceful eccentric contractions during resistance training [138]. Further, the early expression of MGF and/or mechanoreceptor activation increases the synthesis of new myofibrillar proteins within the pre-existing domain. At this point, the radial expansion of the fiber allows some of the split myofibrils as well as a group of satellite cells to fall outside of the myonuclear domain. The lack of myostatin (???) signaling in concert with MGF signaling induces proliferation in the satellite cells that escape the pre-existing domain.

Step 2: Delayed IGF-1Ea signaling induces one proliferating myoblast to differentiate.
Step 3: The fusion of the newly differentiated myoblast allows for the transcription and synthesis of myofibrillar proteins following subsequent exercise stimuli.

Figure 4. The postulated post-exercise molecular phenomena that occur in post-mitotic fibers versus satellite cells following an exercise stimulus:

A) The transient post-exercise mRNA/protein expression and secretion of MGF from post-mitotic fibers acts to signal satellite cells in an IGF-1 receptor-independent paracrine fashion which induces the expression of cyclins and leads to an increase in satellite cell proliferation [27, 76].
B) The delayed mRNA/protein expression of IGF-IEa from post-mitotic fibers and satellite cells (F) acts to possibly increase the expression of MRFs, initiate differentiation, and stimulate protein synthesis in newly differentiated myoblasts [27].
C) The mRNA/protein expression of cell cycle/differentiation-regulating genes likely does not occur in post-mitotic cells [99, 100].
D) The mRNA/protein expression and increased activities of cdk-cyclin genes and concomitant depression in $p27^{Kip1}$ occurs in satellite cells and acts to increase DNA replication and mitosis resulting in an increase in [DNA] (E) within 24 hours following exercise [27, 65]. The variable expression of MyoD during early post-exercise time points indicates that cells which subsequently differentiate may have elevated MyoD concentrations, whereas proliferating cells that do not differentiate may have depressed MyoD concentrations [68]. Finally, the increased expression of $p21^{Cip1}$ within satellite cells may act to assist in the assembly of cdk-cyclin complexes [139], or may act to quell differentiation until the addition of myonuclei to pre-existing fibers are needed.
F) A delayed expression of myogenin, IGF-IEa, and possibly $p27^{Kip1}$ within satellite cells acts to confer myoblast differentiation following proliferation events [20] assuming that myonuclear addition is needed.

Figure 5. Study Design. All workouts (dark gray and black bars) consisted 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. Note that FAMs 1 and 2 will serve to progressively ease all participants into the lower body workouts and that this strategy has been previously performed by Bamman and colleagues [18].

Figure 6. 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 [140] as well as the lack of high molecular weight peaks past the 28S peak (indicative of DNA contamination).

Figure 7. Muscle [DNA] values expressed as means ± SE. There were no between-group or within-group changes over the exercise intervention.

Figure 8. Muscle [total protein] values expressed as means ± SE. There were no between-group or within-group changes over the exercise intervention.

Figure 9. Muscle [myofibrillar protein] values expressed as means ± SE. There were no between-group or within-group changes over the exercise intervention.

Figure 10. Muscle [total RNA] values expressed as means ± SE. $\Delta^{1,2}$: Significant increase in muscle [total RNA] within the young ($p = 0.021$) and old ($p = 0.010$) groups from T3 to T4 denoted by the dashed and solid lines, respectively. Δ^{3}: Significant decrease in muscle [total RNA] within the old group ($p = 0.021$) groups from T3 to T4 denoted by the solid line.

Figure 11. *MGF* mRNA expression values expressed as means ± SE. $\Delta^{1\text{-}3}$: Significant increase in muscle *MGF* mRNA expression within the old group from T2-4 compared to T1 ($p < 0.05$).

Figure 12. *CDK2* mRNA expression values expressed as means ± SE. There were no between- or within-group differences ($p > 0.05$).

Figure 13. *CDK4* mRNA expression values expressed as means ± SE. There were no between- or within-group differences ($p > 0.05$).

Figure 14. *CYCLIN D1* mRNA expression values expressed as means ± SE. ‡$^{1, 2}$: expression values were significantly greater in the older versus younger group at T1-2 ($p < 0.05$). There were no within-group differences ($p > 0.05$).

Figure 15. *IGF-1EA* mRNA expression values expressed as means ± SE. There were no between- or within-group differences ($p > 0.05$).

Figure 16. *MGF:IGF-1EA* mRNA expression values expressed as means ± SE. These values discern the proportion of the IGF-1 gene that is being spliced into *MGF*. $\Delta^{1\text{-}2}$: Significant increase in muscle *MGF:IGF-1EA* mRNA expression within the old group from T3-4 compared to T1 ($p < 0.05$).

Figure 17. MHC_{EMB} mRNA expression values expressed as means ± SE. Δ: Significant increase in muscle MHC_{EMB} mRNA expression within the young group from T4 compared to T1 ($p < 0.05$).

Figure 18. *MYOD* mRNA expression values expressed as means ± SE. Δ: Significant increase in muscle *MYOD* mRNA expression within the young group from T2 compared to T1 ($p < 0.05$).

Figure 19. *P21*^{CIP1} mRNA expression values expressed as means ± SE. There were no between- or within-group differences ($p < 0.05$).

Figure 20. *P27*^{KIP1} mRNA expression values expressed as means ± SE. There were no between- or within-group differences ($p > 0.05$).

Figure 21. Muscle [PCNA] values expressed in ADUs as mean ± SE. There were no between- or within-group differences ($p > 0.05$). The inset figure above provides representative Western blots from a young and old subject over the training intervention.

Figure 22. Muscle [c-met] values expressed in ADUs as mean ± SE. Δ^{1-2}: Significant increase (at T1) and decrease (at T4), respectively, in muscle [c-met] values within the young group compared to T1 ($p < 0.05$). The inset figure above provides representative slot blots from a young and old subject over the training intervention. It should be noted that the secondary antibody solution was confirmed to yield no chemiluminescent signal and that the signal from the slot-blots were generated from primary antibody binding to the target antigen.

Figure 23. Muscle [IGF-1] values expressed in ng/mg skeletal muscle as mean ± SE. There were no between- or within-group differences ($p > 0.05$).

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:_____

Appendix E

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 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 (mononucleosis, diabetes, anemia)? Yes No

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

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

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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, or 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.