# Influence of Aging and Calorie Restriction on Protein Metabolism and microRNA Expression

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**Abstract**

Modulation in the expression of microRNA (miRNA), which are small non-coding RNA that regulate gene expression, has been identified as a potential mechanism controlling age-associated declines in skeletal muscle mass. Recently, miRNA have also been identified to be stable analytes present in circulation (c-miRNA), with alterations in c-miRNA expression profiles suggested to reflect the underlying physiological state of skeletal muscle. The potential for c-miRNA to provide insight into physiological adaptations within skeletal muscle makes them a promising noninvasive marker to assess mechanisms regulating muscle mass. Three investigations were conducted to assess the influence of aging and dietary manipulation on expression of miRNA in circulation and skeletal muscle to determine the functional implications to alterations in miRNA expression profiles. The first investigation sought to determine the influence of aging on c-miRNA expression at rest and following acute resistance exercise in 18 younger (22 ± 1 yrs, $n = 9$) and older (74 ± 2 yrs, $n = 9$) male volunteers. Primary findings revealed that fasting c-miRNA expression profiles were significantly ($P < 0.05$) predictive of aging, with miR-19b-3p, miR-206 and miR-486 distinguishing between age groups. Following resistance exercise, principal component analysis revealed a divergent response in expression of 10 c-miRNA. Using Ingenuity Pathway Analysis to test c-miRNA-to-mRNA interactions in skeletal muscle, it was found that the response of c-miRNA to exercise was indicative of an anabolic response in younger but not older participants. These findings were corroborated with a positive association ($P < 0.05$) observed with the phosphorylation status of p-Akt$^{Ser473}$ and p-70S6K1$^{Thr389}$ and expression of miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, and miR-195-5p. These data provide evidence that alterations of c-miRNA expression with aging may be reflective of underlying molecular mechanisms resulting in age-associated declines in skeletal muscle mass. The second investigation examined the relationship of circulating muscle specific microRNA (c-myomiR; miR-1-3p, miR-133a-3p, miR-133b, miR-206) to whole-body protein synthesis following 28 days of calorie restriction (CR) in 16 older (64 ± 2 yrs) overweight (28.5 ± 1.2 kg·m$^{-2}$) males. Following CR, overall expression of c-myomiR increased ($P < 0.05$) compared to weight maintenance values, with c-myomiR inversely associated ($r = -0.70$, $P < 0.05$) with whole-body protein synthesis. Confirming these results, *in vitro* CR of C2C12 myotubes reduced protein synthesis 2.1 ± 0.2 fold, while myomiR expression in medium increased 2.9 ± 0.1 fold compared to controls. Results from *in vivo* and *in vitro* analysis suggest that increased expression of c-myomiR reflects lower rates of protein synthesis following CR. The final investigation assessed the influence of prolonged (16-wks) 40% CR consuming adequate (10%) or high (32%) protein milk-based diets on skeletal muscle mTORC1 signaling and expression of associated miRNA in 12-wk old male Sprague Dawley rats. Independent of dietary protein intake, CR resulted in lower ($P < 0.05$) muscle protein content, as well as phosphorylation and total Akt, mTOR, rpS6 and p70S6K compared to AL. Despite downregulations in mTORC1 signaling following CR, associated miRNA expression was not altered by either energy or protein intake. Overall, results from these studies suggest that aging results in modulations in c-miRNA profiles that may be further exacerbated by CR, as c-myomiR were upregulated following short-term CR and inversely associated with protein synthesis.

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# I. INTRODUCTION

Skeletal muscle is a critical organ in the human body, comprising approximately 40% of total body mass and contributing significantly to locomotion, metabolism, exercise capacity, and health (1). Due to the skeletal muscles malleable nature, this tissue is capable of adapting its size and metabolism to match demands of various stimuli such as external loading, hormonal balance and nutrient availability (2, 3). With aging, there is an impairment of skeletal muscle plasticity, resulting in a blunted rate of skeletal muscle protein synthesis (i.e., anabolism) following acute exposure to potent anabolic stimuli (4-9). This phenomenon of diminished skeletal muscle anabolism with aging has been termed 'anabolic resistance' (10, 11). Failure to preserve normal anabolic processes while proteolytic (i.e., breakdown) mechanisms are maintained or upregulated with aging results in development of sarcopenia; age-associated decline in skeletal muscle mass and function (12, 13). Over time, the progressive loss in muscle mass can compromise an individual's quality of life, leading to loss of independence and diminished health-span.

To minimize declines in skeletal muscle mass and mobility with aging, an understanding of the underlying molecular processes regulating anabolism is crucial to determine potential therapeutic targets. Recently, microRNA (miRNA; small non-coding RNA, approximately 18-25 nucleotides in length) have been identified as regulators of exercise-induced adaptions (14-16) and aging (17-19) within skeletal muscle. Furthermore, miRNA have been identified as stable analytes present in circulation (c-miRNA) (20). In response to physiological states (e.g., exercise and disease) that impact skeletal muscle mass, alterations in c-miRNA profile may be reflective of the underlying condition of the tissue (21, 22). The potential for c-miRNA to provide insight into of

physiological processes in skeletal muscle makes them a promising noninvasive marker. However, little is known regarding the influence of aging and dietary intervention on c-miRNA profiles. Whether c-miRNA expression can be used as noninvasive markers to assess adaptions of physiological processes resulting in age-associated declines in skeletal muscle remains undetermined. Furthermore, whether alterations in c-miRNA profiles in response to exercise or dietary interventions reflect alterations in anabolic signaling pathways that regulate muscle mass has not been determined.

## II. OBJECTIVES AND HYPOTHESES

**STUDY 1:**

**Aim 1: Assess the influence of aging on c-miRNA expression under resting fasted conditions.**

***Hypothesis:*** *Expression of c-miRNA profiles will distinguish between younger and older participants.*

**Aim 2: Determine the influence of an acute bout of resistance exercise on changes in c-miRNA expression, and establish if c-miRNA are predictive of exercise-induced adaptions.**

***Hypothesis:*** *Acute resistance exercise will stimulate a divergent response in c-miRNA expression between younger and older participants. Altered expression of c-miRNA following acute resistance exercise will be associated with changes in phosphorylation of anabolic signaling proteins.*

**STUDY 2:**

**Aim 1: Assess the influence of short-term calorie restriction on skeletal muscle associated c-miRNA expression.**

***Hypothesis:*** *Short-term calorie restriction will increase the expression of skeletal muscle associated c-miRNA compared to weight maintenance.*

**Aim 2: Assess the association of skeletal muscle associated c-miRNA expression to whole-body protein turnover following short-term calorie restriction.**

***Hypothesis:*** *Following short-term calorie restriction an inverse correlation will be observed between whole-body protein turnover and skeletal muscle associated c-miRNA expression.*

**STUDY 3:**

**Aim 1: Determine the effects of prolonged CR and high protein diets on mTORC1 signaling, and assess whether alterations in mTORC1 signaling influence skeletal muscle mass.**

***Hypothesis:*** *mTORC1 signaling protein activity will be downregulated with CR adequate protein intake resulting in declines in skeletal muscle mass. High protein diets with CR feeding will maintain mTORC1 signaling and skeletal muscle mass.*

**Aim 2: Examine the potential regulatory role of skeletal muscle miRNA expression on mTORC1 signaling.**

***Hypothesis:*** *Modifications in mTORC1 signaling proteins in response to CR and dietary protein intake will be associated with altered expression of regulating miRNA.*

## III. LITERATURE REVIEW

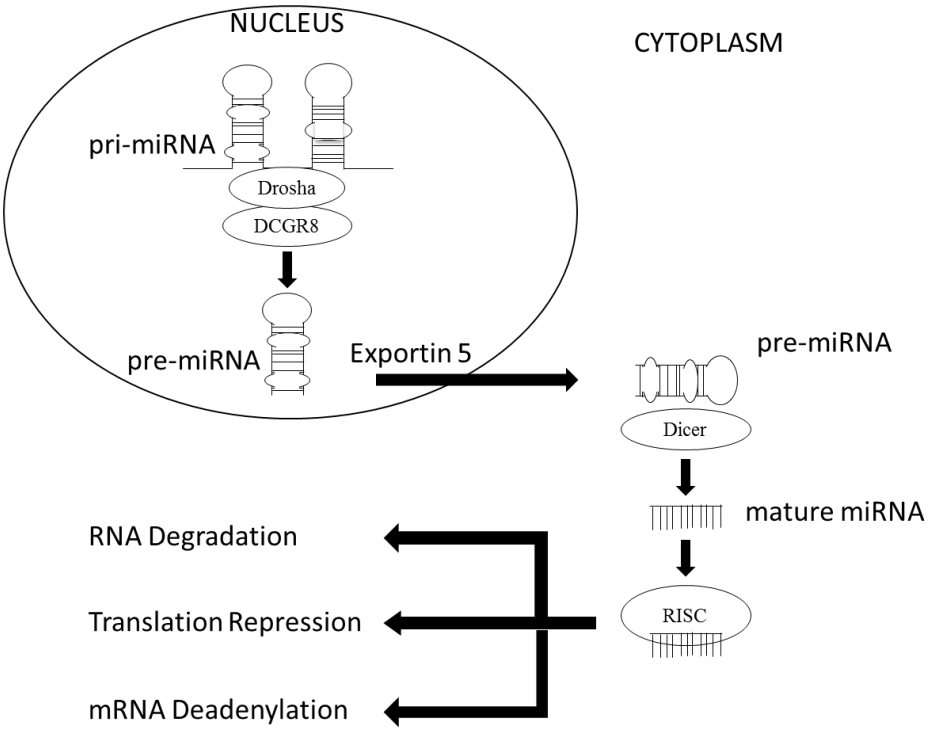
### a. microRNA biogenesis and function

Biogenesis of miRNA begins in the nucleus of the cell (**Figure 1**), where RNA polymerase II transcribes primary-miRNA (pri-miRNA), consisting of thousands of nucleotides with stem-loop structures (23). The enzyme Drosha, a member of the ribonuclease (RNase) III superfamily of double-stranded RNA-specific endoribonuclease, together with DiGeorge syndrome critical region gene (DGCR8), cleaves the stem-loop structure of the pri-miRNA to form precursor miRNA (pre-miRNA) (24, 25). Conversion of pri-miRNA to pre-miRNA is a critical step, as it is site-specific, dictating the sequence of the mature miRNA (26). The pre-miRNA translocates out of the nucleus into the cytoplasm by small RNA transporter Exportin 5, which is a GTP dependent process (27). Once in the cytoplasm, pre-miRNA is processed by Dicer, another enzyme in the RNase III family, to form mature miRNA (28). One strand of the mature miRNA is bound by Argonaute, a protein that directly binds to miRNA, forming a protein complex called RNA-induced silencing complex (RISC), while the second strand is degraded (29, 30).

In the cytoplasm the RISC complex allows miRNA to bind to target mRNA, resulting in post-transcriptional modifications that repress the translation of protein (31, 32). Regulation of protein expression occurs through both perfect and imperfect binding to the 3' untranslated regions of miRNA to mRNA. Perfect binding of miRNA-to-mRNA leads to cleavage or degradation of the mRNA, while imperfect binding results in translational repression (33). Through these mechanisms of negative inhibition miRNA control the regulation of gene expression. miRNA-dependent gene regulation is a complex process,

as one miRNA can regulate hundreds to thousands of genes (34). The ability for one miRNA to inhibit the expression of a large number of genes allows a single miRNA to repress several mRNA in a common biological pathway, resulting in robust regulation of an entire molecular process (35, 36). Additionally, one gene can be targeted by multiple miRNA, resulting in cooperative / redundant regulation of a signal molecular process (34). Seeding of miRNA into protein coding genes not only serves to regulate protein translation, but also regulates the expression of the miRNA, either inhibiting or elevating its expression, resulting in a complex feedforward / feedback network (34, 37, 38). Through these mechanisms of regulation, miRNA have a critical role in the development and maintenance of physiological functions and disease processes.

**Figure 1.** microRNA biogenesis



## b. microRNA regulation of skeletal muscle mass

### i. Myogenesis and regeneration

Generation of muscle, known as myogenesis, is fundamental to not only the synthesis and development of new skeletal muscle fibers, but is also a critical process for regeneration and hypertrophy of adult skeletal muscle fibers (39, 40). The complex, multi-stage process regulating development and regeneration of skeletal muscle requires coordination of multiple transcription factors to proliferate and differentiate myogenic precursors to mature myofibers (41). Ultimately this process determines muscle fiber number, type/phenotype, and mass/size (42).

Proliferation, the initial phase of myogenesis, occurs in embryonic cells, as founder stem cells (i.e., non-specified cells capable of self-renewal and becoming tissue/organ-specific cells) differentiate into myocytes and progress during fetal development to become mature myofibers (43, 44). Postnatal juvenile skeletal muscle stem cells (i.e., satellite cells) continue to proliferate, relocating between the basal lamina of the myofibers, where they are essential for growth (45). These juvenile satellite cells transition to adult mitotically quiescent satellite cells, the primary source of myoblasts required for muscle repair and regeneration (46, 47). Quiescent satellite cells, located at the periphery of myofibers, residing between the sarcolemma and the basal lamina (45, 48), are critical for the maintenance of muscle mass as they self-renew through asymmetric division, resulting in an undifferentiated mother cell and a committed progeny, myogenic precursor cell, to synthesis and repair myofibers (47).

Paired-box (Pax) transcription factors Pax3 and Pax7, which mark myogenic progenitor cells, are important components of myogenesis and regenerations, regulating

myogenic regulatory factors (MRFs), a family of basic-Helix-Loop-Helix transcription factors that bind to the promoter region of several muscle specific genes to initiation proliferation and differentiation (43, 49). Downstream transcription factors are targeted by Pax3 and Pax7 are MyoD, myogenic factor 5 (Myf5), Myf6, and myogenin (**Figure 2**), which control commitment of satellite cells to myoblast formation and differentiation and fusion of myocytes to myofibers (50-54). The earliest markers of myogenic cells are Myf5 and MyoD, which are involved in the commitment of proliferating satellite cells to myogenesis and regeneration (55). These two MRFs appear to have somewhat redundant functionality, as knocking out either of these genes still allows for myogenesis and regeneration, albeit at limited capacity (55). If both genes are silenced, however, myocyte formation does not occur. Following activation, satellite cells exhibit a co-expression of Pax7 and MyoD (56). Here, MyoD regulates cell cycling through expression of the gene Cdc6, which is involved in DNA replication, allowing for propagation of cell cycling form the G1 phase to the S phase (57). Following proliferation of myoblasts, an alteration in the ratio of Pax7:MyoD occurs, where Pax7 expression decreases and MyoD expression increases (53). In the terminal stage of differentiation, persistent elevations in MyoD are associated with increased expression of myogenin, a transcription factor required for the fusion of myoblasts to complete the formation of new or repair of previously existing muscle fibers (58). Alternatively, satellite cell progeny can maintain Pax7 and Myf5 expression, while suppressing MyoD and myogenin (59). This pathway allows for continuance of the myogenic lineage, renewing the quiescent satellite cell pool. Maintenance of satellite cells provides for future myogenesis in response to exercise or muscle injury.

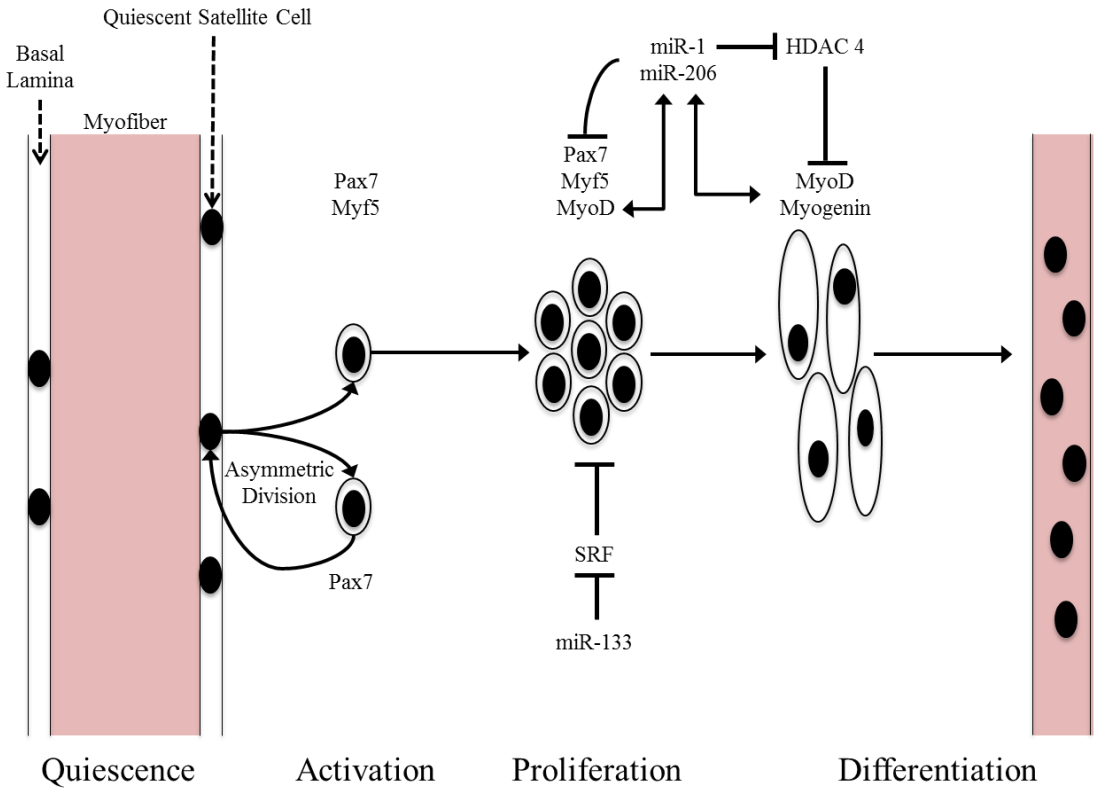
Expression of miRNA has recently been identified as an important regulatory factor involved in myogenesis and regenerations. Using tissue-specific Dicer deletion in mice, O'Rourke et al. (60) discovered that removal of this key enzyme required for maturation of miRNA downregulated overall miRNA expression in skeletal muscle, resulting in reductions in the number of myofibers, elevations and apoptosis, and eventually death of the animal (60). Negative consequences of diminished miRNA expression in skeletal muscle can likely be attributed to reductions in muscle-specific miRNA, termed myomiR. This class of miR primarily refers to miR-1, miR-133a, miR-133b, and miR-206 (61), which constitute nearly 25% of all miRNA expressed in the skeletal muscle (62-64), and are required for proliferation and differentiation to synthesis and repair skeletal muscle fibers (65, 66).

The ability for MRFs to shuttle satellite cells through proliferation to differentiation has recently been shown to be dependent on complex feed-forward and feedback mechanism with specific myomiR (**Figure 2**). Alterations in the ratio of Pax7:MyoD during differentiation is reliant on expression of miR-1 and miR-206. There is an inverse association between Pax7 and miR-1/miR-206 expression during differentiation of myogenic progenitor cells (67-69). Repression of miR-1 and miR-206 expression in cell culture models result in an elevated expression of Pax7, promoting proliferation while diminishing differentiation and thus delaying myogenesis (68, 69). When miR-1 and miR-206 are overexpressed there is a reduction in Pax7 protein content, resulting in a downregulation in proliferation and an upregulation in muscle progenitor cell differentiation (68). MyoD and myogenin are the transcription factors regulating increased expression of miR-1 and miR-206 during differentiation by binding to

upstream genomic regions to induce their expression (65, 70). Through this induction of miR-1 and miR-206 expression, MyoD and myogenin promote their own expression through the inhibition of Pax7. Given the importance of Pax7 in maintaining satellite cell pools and inhibiting differentiation, expressions of miR-1 and miR-206 have a crucial mechanistic role in determining the fate of satellite cells, either being used for renewal the satellite cell pool or lead to terminal differentiation into myotubes.

Further regulation of myogenesis by miR-1 and miR-206 occurs through repression of histone deacetylase 4 (HDAC4), an inhibitor of differentiation (71). HDAC4 suppresses myocyte enhancer factor-2 (MEF2) dependent transcription, which is required for the activation of MRFs (72). Interestingly, the repression of HDAC4 by miR-1 begins a feed-forward loop, where miR-1 allows for enhanced expression of MEF2, the transcription factor of miR-1, resulting in a further upregulation of its expression to further enhance myoblast differentiation (67). Additionally, miR-206 promotes differentiation by targeting the p180 subunit of DNA polymerase α to diminish cell cycle to allow for commitment to differentiation (73). While miR-1 and miR-206 repress proliferation and promote differentiation, miR-133 increases proliferations of myoblasts and inhibits differentiation (74-76). Expression of miR-133a is upregulated by serum response factor (SRF), a transcription factor that regulates cell cycling, apoptosis, cell growth, and cell differentiation. Increased miR-133a expression initiates a negative feedback loop, as miR-133a targets SRF to repress its expression, allowing for enhanced proliferation (67). Together these data show that myomiR are critical to maintain and potentially enhance myogenesis and regeneration to optimize skeletal muscle health.

**Figure 2.** Function of myomiR in myogenesis and regeneration




## ii. Muscle hypertrophy

Skeletal muscle hypertrophy is achieved when the rate of muscle protein synthesis exceeds the rate of muscle protein breakdown, resulting in a positive net protein balance for a sustained period of time (77). The mechanisms regulating protein synthesis and breakdown are highly controlled, integrated systems of intracellular signaling proteins that modulate gene transcription, translation, and post-translational modifications (78, 79). The mechanistic target of rapamycin complex 1 (mTORC1) is the master regulator of the intracellular signaling pathway controlling protein synthesis (80). Activation of the mTORC1 pathway can be initiated by energy and nutrient availability (e.g., amino acids), growth factors (e.g., insulin like growth factor 1), and mechanical load. Upregulation of mTORC1 propagates the activation of its downstream targets p70

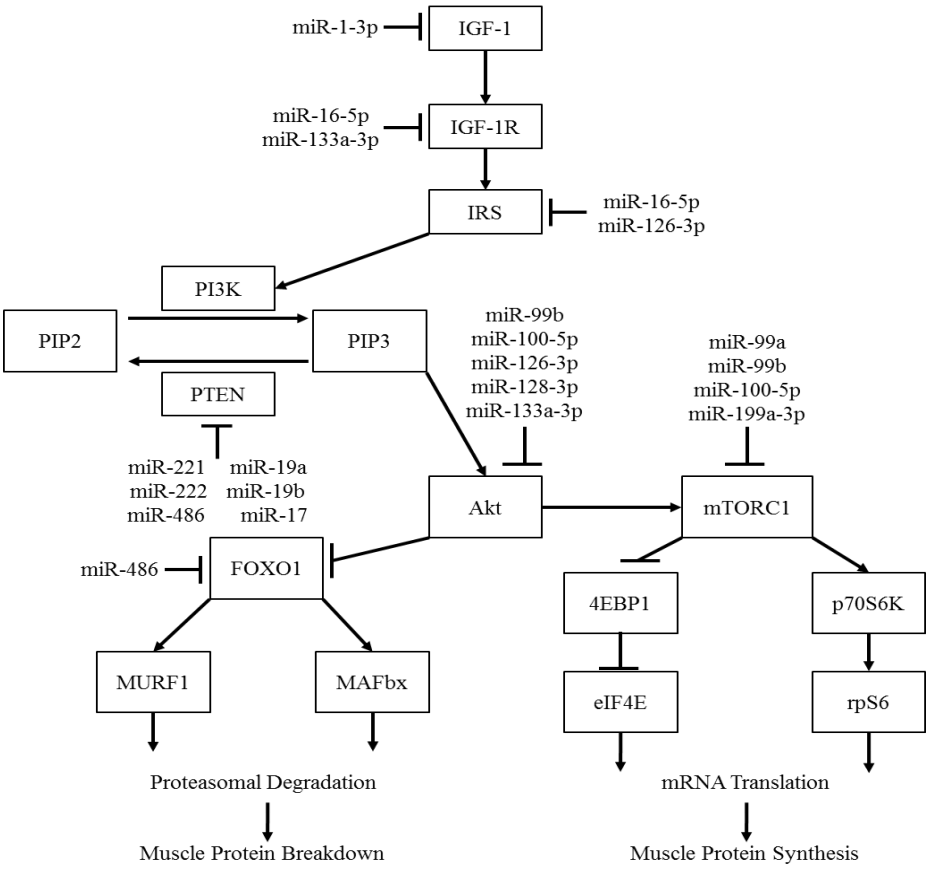
ribosomal S6 kinase (p70S6K1) and ribosomal protein S6 (rpS6), which regulate mRNA translation to enhance protein synthesis (81, 82). Additionally, mTORC1 phosphorylates eukaryotic initiation factor 4E-binding protein (4E-BP1), which causes 4E-BP1 to dissociate from eukaryotic translation initiation factor 4E (eIF4E) (83). Upon being released by 4E-BP1, eIF4E can bind with eIF4G and eIF4G to form a multi-subunit complex (eIF4F) that that is critical for cap-dependent mRNA translation (84). This signaling cascade culminates with the increase of mRNA translation initiation and elongation to induce muscle protein synthesis (**Figure 3**).

Regulation of muscle protein breakdown functions primarily through the ubiquitin proteasome system (85). Upregulation of protein breakdown can result from diminished energy availability and increased inflammation/stress. Muscle-specific E3 class of ubiquitin ligases, muscle atrophy F-box (MAFbx) and muscle RING finger-1 (MuRF1), regulate myofibrillar proteolysis through ubiquitination (86). Transcript factor forkhead box O 1 (FOXO1) is the upstream regulator of MAFbx and MuRF1 activity. Dephosphorylated FOXO1 translocates to the nucleus of the myofiber to mediate increased expression of these E3 ubiquitin ligases, resulting in ubiquitination of myofibrillar proteins, which are transferred to the 26S proteasome for subsequent degradation (87). While mTORC1 and FOXO1 signaling result in divergent outcomes, cross talk exists between these anabolic and catabolic pathways. A primary point of integration occurs at protein kinase B (Akt), an upstream mediator of both mTORC1 and FOXO1 (88). When Akt is upregulated through IGF-1 signaling, mTORC1 is activated to increase rates of protein synthesis, while FOXO1 is phosphorylated to inhibit its translocation into the nucleus resulting in diminished protein breakdown (88).

There is a growing body of evidence that miRNA have a significant impact on skeletal muscle growth, with several miRNA participating in the regulation of signaling proteins within the mTORC1 and FOXO1 signaling cascades (**Figure 3**). In muscle, miR-1, miR-133a-3p, and miR-199a-3p target IGF-1 and IGF-1R, blunting rates of protein synthesis (89, 90). During periods of muscle growth, induced by mechanical load, miR-1 and miR-133a expression is downregulated to allow for activation of mTORC1 signaling through IGF-1, resulting in increased rates of protein synthesis (62). In muscle atrophy, miR-199a-3p expression is increased (91), with this overexpression resulting in impairment of muscle hypertrophy, diminishing phosphorylation of Akt and mTORC1 (92). Additionally, miR-99a, miR-99b and miR-100-5p influence cellular growth by both directly and indirectly mediating translation of mTORC1. Specifically, increased expression of miR-99a and miR-99b inhibit transcription of mTOR, while miR-100-5p targets both Akt and mTOR, resulting in diminished total protein content and hypertrophy (93, 94). Furthermore, it is possible that miRNA play an important role in the shifting of intracellular signaling from anabolism to catabolism. Expression of the upstream inhibitor of Akt, phosphatase and tensin homolog (PTEN) is diminished by miR-221, miR-222, and miR-486 to promote cellular growth (95, 96). Furthermore, the miR-17~92 cluster, which contains 7 miRNA, participates in alterations in Akt-mTOR signaling. Similar to miR-221 and miR-222, miRNA in the miR-17~92 cluster (miR-17-5p, miR-19a-3p and miR-19b-3p) inhibit PTEN, promoting Akt-mTOR signaling (97). Increased Akt activity due to alterations in miRNA expression may not only promote synthesis, but diminish protein breakdown as well, resulting in a positive protein balance. Upregulation of miR-486 activates Akt and diminishes FOXO1 protein expression.

Diminished FOXO1 by miR-486 results in reductions in transcription of atrophy proteins MAFbx and MuRF1, potentially minimizing muscle protein breakdown (95, 98). Important to note, much of the information regarding miRNA regulation of the abundance of molecular signaling proteins crucial to the regulation of anabolism and catabolism has been conducted in cell culture experiments and disease models. Little is known regarding the influence of specific miRNA on regulation of skeletal muscle mass with aging and in response to exercise and diet interventions in humans and animals.

**Figure 3.** Interaction of microRNA in muscle hypertrophy and atrophy signaling pathways



### c. Aging and skeletal muscle microRNA expression

Mechanical load placed on skeletal muscle during resistance exercise is well known to active anabolic intracellular signaling pathways, increasing the rate of muscle protein synthesis and, over time, leading to gains in muscle mass (79). Aging results in a blunted anabolic response to an acute bout of resistance exercise (99), diminishing an individual's ability to maintain skeletal muscle mass and function (100). As miRNA target molecular pathways governing skeletal muscle anabolism and proteolysis, dysregulation in expression of miRNA with aging may provide novel insight into their use as potential therapeutic targets to overcome this 'anabolic resistance.'

Only a few investigations have illuminated the potential role of dysregulation in miRNA expression on age-associated declines in skeletal muscle mass (101-103). Under resting conditions, aging increases expression of the miRNA Let-7b and Let-7e, diminishing the expression of cell cycle regulatory genes CDK6, CDC25A, and CDC34 within skeletal muscle (102). Downregulation of these genes reduces satellite cell turnover and, thus, skeletal muscle renewal/regeneration through inhibition of Pax7 gene expression. The lack of self-renewal observed with aging due to alterations in miRNA has the potential compromise skeletal muscle integrity by diminishing mechanisms to repair damaged muscle fibers. However, whether alterations in miRNA expression with aging result in cellular senescence has yet to be determined.

Altered response of miRNA expression to an acute anabolic stimulus such as resistance training may also contribute to age-associated losses in skeletal muscle mass. Following performance of knee extension exercise fixed at 70% of participant's one repetition maximum (RM), expression of miR-1 was reduced in young participants, with

no change observed in older individuals (101). Divergent responses in miR-1 expression with aging likely indicates a lack of an anabolic response to the bout of resistance exercise. In animal models, periods of muscle hypertrophy brought on by functional overload, where the gastrocnemius and soleus were removed to induce plantaris anabolism, result in diminished miR-1 expression (62). As miR-1 inhibits IGF-1 in skeletal muscle, reductions in miR-1 expression during periods of hypertrophy suggest potential activation of the mTORC1 pathway through IGF-1 signaling. In agreement with these findings, our laboratory (103) recently observed that following a single bout of resistance exercise, expression of 60 miRNA assessed in skeletal muscle were not altered in older participants, while younger participants experienced a significant reduction in the expression of 16 of these 60 miRNA. The absence of exercise-induced miRNA regulation with aging was accompanied by a blunted gene transcription response, and diminished activation of the mTORC1 signaling cascade compared to younger participants (104). Impairment of resistance exercise-induced alterations in skeletal muscle miRNA and mRNA expression, as well as diminished phosphorylation of mTORC1 signaling with aging suggests a potential link governing 'anabolic resistance.'

To further examine whether altered miRNA expression was a potential mechanistic target for diminished muscle mass with aging, principal component analysis was conducted on miRNA with differing expression between younger and older to determine which miRNA distinguished aging (103). This analysis identified miR-126-3p as a potential target influencing the divergent anabolic response to resistance exercise with aging. To test the role of miR-126-3p on regulation of molecular pathways controlling skeletal muscle anabolism, *in vitro* analysis was performed, manipulating the expression

of miR-126-3p through transfection of miR-126-3p inhibitor or mimetic for 24-hrs in myocytes and myotubes. Inhibition of miR-126-3p protein content of insulin receptor substrate 1 increased 50%, while FOXO1 decreased 25% compared to control myocytes. Additionally, when miR-126-3p was overexpressed, myogenic regulators MyoD and Myf5 were 60% and 50%, respectively, lower compared to controls. Following 30 min exposure to IGF-1 in myotubes upregulation of p-Akt$^{Ser473}$ was greater in miR-126-3p inhibited myotubes compared to control. Similarly, a downstream target of mTORC1, p-rpS6$^{Ser240/244}$ was activated to a greater extent in miR-126-3p inhibited myotubes compared to controls. Together, findings from *in vivo* and *in vitro* analysis identify miR-126-3p dysregulation with aging as a novel regulator suppressing skeletal muscle regeneration and growth following exercise-induced adaptions within skeletal muscle.

In agreement with findings from our laboratory, high-throughput analysis of 754 miRNA identified 26 miRNA that were differentially expressed with aging in response to resistance exercise, or a combination of the two (105). Top cellular function of these miRNA were determined using Ingenuity Pathway Analysis. This bioinformatics analysis revealed that 6 (miR-99a-5p, miR-99b-5p, miR-100-5p, miR-149-3p, miR196b-5p, and miR-199a) of these 26 miRNA were validated to target proteins within the Akt-mTORC1 signaling cascade. As described above, members of the miR-99/100 family are of particular interest, as these miRNA directly target mTOR to suppress protein synthesis and anabolism. Specifically, following acute resistance exercise miR-99b-5p and miR-100-5p expression were diminished in young but not old participants. Again, the lack of response in expression of miRNA associated with regulation of anabolic signaling proteins with aging in skeletal muscle indicate dysregulation in miRNA expression

following acute anabolic stimulus may contribute to age-associated declines in skeletal muscle mass.

**d. Circulating microRNA expression**

Assessment of miRNA expression within skeletal muscle has yielded valuable insight into their potential role as therapeutic targets to attenuate age-associated declines in muscle mass. However, *in vivo* analysis of skeletal muscle miRNA in humans requires invasive muscle biopsies, which may cause discomfort and limit tissue available for analysis, especially in an older and frail population. In an attempt to minimize participant discomfort and capture larger datasets to enhance our understanding of underlying mechanisms regulating diminished muscle mass and function with aging, determination of noninvasive markers reflective of skeletal muscle physiology are warranted.

While miRNA have been shown to function in the cell where they are transcribed, recently, it has been reported that an alternative fate exists, where rather than remaining in the cytoplasm of the cell, miRNA can be packaged and exported into the circulation (21). The profile of these circulating miRNA (c-miRNA) has been shown to be altered in response to physiological states that impact skeletal muscle mass, such as disease states (106-108) and physical activity (109-111). Important to note, altered expression of c-miRNA under these conditions are reflected in the muscle-specific myomiR, which allows some conclusions to be drawn about tissue physiology of donor cells. The ability of c-miRNA expression to provide insight into changes of physiological processes in skeletal muscle makes them a promising noninvasive marker to assess mechanisms regulating age-associated declines in skeletal muscle mass, such as 'anabolic resistance.'

Presence of miRNA in the circulation can be the result of multiple mechanisms and transporters. Within the cytoplasm, membrane-derived vesicles (exosomes and microvesicles) can take up pre and mature miRNA, where they can then be released into the circulation to be transferred to recipient cells (112). In addition to exosomal and microvesicle transportation, c-miRNA are actively transported in RNA binding protein (Argonaute2) and lipoproteins (high density lipoprotein (HDL) and low density lipoprotein (LDL)) (113-116). Furthermore, miRNA can be passively present in circulation in apoptotic bodies that have been shed by tissues (117). Though the mechanistic regulation of secretion of miRNA into circulation has yet to be established, it has been suggested that neutral sphingomyelinase 2 (nSMase2), the rate limiting enzyme for synthesis of ceramide, has a functional role in triggering the secretion of exosome encapsulated miRNA (118). Once released into circulation, c-miRNA can be taken up by recipient cells to inhibit transcription of target genes (119). Similar to their secretion, the mechanism involved in the uptake of exosome bound c-miRNA by recipient cells remains elusive. Potential mechanism to remove miRNA from circulation have recently been highlighted by Hackl *et al.* (120), and include endocytosis (121), fusion to the plasma membrane (122), scavenger receptor uptake (115), or interaction at the cellular surface to alter intracellular signaling (123).

Though the exact mechanism by which c-miRNA influence cellular processes is still unknown, it has been observed that alterations in c-miRNA profile reflect the underlying physiological state of the tissue (124). In disease states that are known to negatively impact skeletal muscle, such as COPD, type 2 diabetes, Duchenne muscular dystrophy (DMD), and rhabdomyosarcoma, there is an upregulation in skeletal muscle-specific

circulating myomiR (c-myomiR; miR-1, miR-133a, miR-133b, miR-206), compared to healthy controls (106, 125, 126). Specific to DMD, several investigations conducted in both animal and human models have consistently reported expression of c-myomiR were elevated in the disease state (69, 107, 127-129). Comparing c-myomiR to classic muscle disorder biomarkers creatine kinase, myoglobin and lactate dehydrogenase using receiver operating characteristic (ROC) curve statistical analysis in patients with DMD, multiple investigations have reported that c-myomiR are more sensitive and specific to presence of the disease (107, 128, 129).

**e. Aging and circulating microRNA**

Aging is a multifactorial process that disrupts normal cellular homeostasis, resulting in declines in skeletal muscle mass and function. Understanding physiological alterations in aging muscle is critical to determine therapeutic interventions to mitigate losses in muscle mass in hopes to maintain physical function and independence. However, attainment of muscle samples, especially in older frail individuals, can be difficult due to lower amounts of the tissue and high infiltration of intermuscular fat (130). As such, there is a need to identify noninvasive biomarkers of aging that are suggestive of the underlying physiological state of skeletal muscle. Given that c-miRNA have been shown to reflect skeletal muscle physiology in diseases states that negatively impact muscle mass and function, it stands to reason that assessment of c-miRNA profiles with aging could serve as a biomarker for skeletal muscle adaptions.

Presently, very little is known about alterations in c-miRNA expression in aging, with even less understood about their functional implications. To date, only a few studies (131-135) have been conducted investigating aging and c-miRNA expression, with the

most in-depth analysis conducted by Zhang *et al.* (135). In this study, investigators assessed the alteration of c-miRNA profiles in 173 males and females, broken down into four age groups, where mean ages were 22 yrs, 40 yrs, 59 yrs, and 70 yrs old. Using Taqman® probe-based RT-qPCR assay 853 miRNA were assessed. Threshold cut points were set to isolate miRNA that investigators believed to be representative of significant alterations with aging. Thresholds were set that c-miRNA expression must have a 2-fold change with aging, be statistically significant ($P < 0.05$), and have a cycle threshold (Ct) of < 35. Using these criteria, aging resulted in altered expression of 13 c-miRNA in females and 22 c-miRNA in males. Between sexes, 10 common c-miRNA were similarly altered, with let-7b, miR-92a, miR-222 and miR-375 being upregulated, while miR-19b, miR-29b, miR-106b, miR-130b, miR-142-5p and miR-340 were progressively downregulated with aging. Bioinformatics analysis was then conducted to determine potential targets of these age-altered c-miRNA. Unfortunately, only major protein classes (e.g., transcription factors, protein kinase, etc.) were reported as potential targets, with no assessment of miRNA interactions to specific molecular markers within signaling pathways. Furthermore, bioinformatics analysis was only performed on individual miRNA, and not on a combination of all 10 age-altered c-miRNA to determine if there were any overlapping targets. While this investigation shows a clear alteration in c-miRNA profiles with aging, the functional implication of these changes remains unclear.

Future investigations assessing the influence of aging on c-miRNA expression must attempt to provide a more comprehensive analysis of the physiological ramification of altered c-miRNA profiles. Specifically, bioinformatics analysis should focus on specific molecular targets of miRNA to determine potential signaling pathways. Additionally,

when there is a divergent expression in multiple c-miRNA with aging, bioinformatics analysis should be conducted on all miRNA to determine common targets. As one miRNA can target hundreds genes, showing several c-miRNA converging on a common gene and/or molecular pathway would strengthen any potential functional implication. To ensure that c-miRNA are reflective of skeletal muscle adaptions, assessment of miRNA expression within the tissue, as well as analysis of target molecular pathways is crucial in early investigations to appropriately model c-miRNA profiles. Finally, while aging has been observed to alter c-miRNA profiles, little is known regarding the influence of exercise and dietary manipulation on c-miRNA expression with aging. Determining how c-miRNA expression is influenced by factors that impact skeletal muscle mass, such as resistance exercise and calorie restriction, would allow for more accurate modeling for how c-miRNA profiles reflect acute and sustained modifications within skeletal muscle.

### f. Considerations when assessing circulating microRNA

While identification of altered c-miRNA profiles under various physiological conditions has resulted in much work attempting to determine their function and use as non-invasive biomarkers, there are several strengths and limitations that must be considered when measuring c-miRNA (**Table 1**).

**Table III-1.** Potential of circulating microRNA as noninvasive markers of physiological state at tissue level

| **Strengths** | **Weaknesses** |
|---|---|
| Non-invasive measurement | Inter-individual variation |
| Stable analyte | Lack of standards |

| | |
|---|---|
| Reproducible | Multiple tissues of origin |
| Associated with clinical outcomes | Methodological limitations to isolate and |
| Conserved among different species | determine miRNA transporter |
| Specific to tissues or biological stages | Function undetermined |
| | Susceptible to cellular contamination |

Of note, c-miRNA share many of the characteristics of a biomarker, having been shown to be remarkably stable due to being transported within extracellular vesicles, reproducible, and predictive of clinical outcomes in disease states (136). Furthermore, the hormone-like manner that miRNA are released by donor cells and taken up by adjacent and distal cells suggest that c-miRNA are not passive downstream effects of upstream cellular adaptions, but rather serve a functional role in cellular adaptions. With several miRNA targeting the same gene or molecular pathway, multiple miRNA with similar functions can be assessed to create a profile of c-miRNA, rather than relying on one analyte. Though there is potential to use c-miRNA as a biomarker, the field of c-miRNA research is still in its infancy, and a great deal still unknown. Controlling for factors that may result in variation in c-miRNA expression between individuals is critical to ensure these profiles are truly reflective of what investigators are assessing. Population-based analysis is required to determine standards for level of c-miRNA expression to be used as biomarkers. While tissue specific miRNA in circulation allows for inference to be made about the physiological state of that tissue, miRNA can originate from multiple tissues. Improvement in methods to assess c-miRNA expression to

determine what tissue they originated from, as well as their mechanism of transport, is critical to capture the functional implications of alterations in c-miRNA profiles.

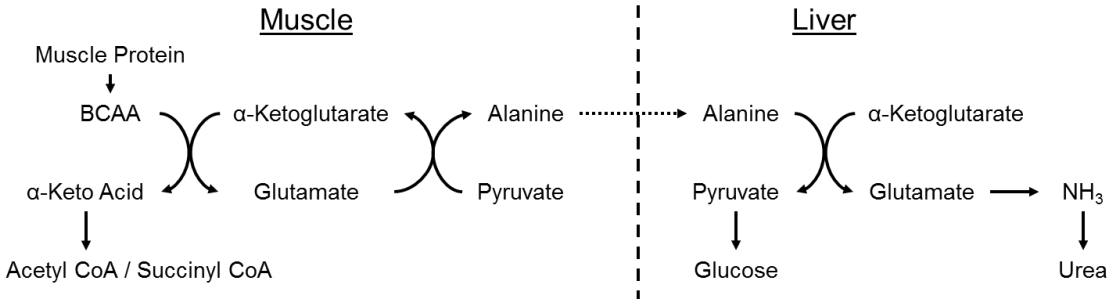
**g. Weight loss and aging**

With 35% of adults age 60 and over considered obese and medical cost attributed to obesity rising from $78.5 billion in 1998 to $147 billion in 2008, and with per capita spending being greatest for Medicare recipients, obesity in older adults is a major health and economic concern (137, 138). Despite evidence of modest weight loss (5-10% body weight) improving body composition (e.g., reductions in fat mass), and metabolic and cardiovascular parameters (139, 140), controversy exists regarding weight loss for older adults due to unintentional losses of skeletal muscle mass, which may compromise physical function and increase frailty (141).

Reductions in skeletal muscle mass brought about by calorie restriction are the result of altered protein turnover (142-144). At the initiation of calorie restriction, there is an upregulation in whole-body protein proteolysis and oxidation (145, 146), with the activity of genes and enzymes that regulate gluconeogenesis and nitrogen metabolism upregulated to increase reliance on amino acids for substrate to be used for energy production and maintenance of glucose homeostasis (147, 148). Specifically, there is an increased synthesis of alanine and glutamine in skeletal muscle through catabolism of branched-chain amino acids (BCAA) leucine, valine, and isoleucine to serve as nitrogen transporters to the liver. Once taken up by the liver, alanine and glutamine are broken down to be used as precursors for gluconeogenesis, the carbon backbone used for pyruvate formation while their amino group forms urea, resulting in increased nitrogen excretion with calorie restriction (149). Mobilization of endogenous amino acids for

energy production results in a negative nitrogen/protein balance indicative of reductions in skeletal muscle mass (78). Following the initial phase of calorie restriction (>14 days) adaptations will occur where there is a downregulation in whole-body protein turnover to spare energy and amino acids stores by diminishing proteolysis and oxidation (150, 151). Though turnover is reduced, individuals will remain in negative protein balance until a new homeostasis is achieved (152).

**Figure 4.** Alterations in amino acid metabolism during weight loss




Calorie restriction-induced reductions in skeletal muscle mass is also believed to be due to alterations in mTORC1 signaling, as cellular energy status is a critical regulator of this pathway. With calorie restriction, there are reductions in cellular adenosine triphosphate (ATP) and elevations in adenosine monophosphate (AMP) (153). Increased AMP:ATP ratios initiate 5' AMP-activated protein kinase (AMPK) activity, a central regulator of energy metabolism (154). The mTORC1 and AMPK signaling pathways have divergent responses to energy status, sufficient energy/nutrient availability activates mTORC1 to promote cellular growth and proliferation, whereas AMPK is activated by lack of energy/nutrients, inhibiting ATP consuming anabolic pathways, while promoting oxidative metabolism to stimulate ATP production (155). Inhibition of anabolism by AMPK occurs through interactions with mTORC1. Upregulation of AMPK inhibits

RAPTOR, a protein in the mTORC1 complex that enables translocation of mTORC1 to the lysosome for activation (156). AMPK can also downregulate mTORC1 through stimulations of Tuberous Sclerosis 2 (TSC2). Increased activity of TSC2 inhibits Ras homolog enriched in brain (RHEB), a protein critical to the activation of mTORC1 (157). Overall, it appears that low cellular energy availability during calorie restriction blunts anabolic mechanisms in favor of ATP yielding catabolic pathways to maintain energy balance and promote cell survival.

**h. Calorie restriction and skeletal muscle and circulating microRNA expression**

Relatively little is known regarding the influence of calorie restriction on skeletal muscle and circulating miRNA expression. To date only one study on non-human primates has been conducted assessing the influence of calorie restriction on skeletal muscle miRNA expression. This study was conducted in aging rhesus monkeys to assess if calorie restriction could revert miRNA profiles to those observed in younger animals (158). RNA seq demonstrated that aging resulted in an alteration of 35 miRNA. Ingenuity Pathway Analysis identified that these divergent c-miRNA are involved in connective tissue and skeletal muscle disorders, cell cycle, inflammatory diseases and response, and skeletal muscle system development and function. Lifelong calorie restriction mitigated age-induced modification expression of several miRNA to include miR-181a, miR-451, miR-144, miR-18a and miR-15a. For regulation of skeletal muscle mass, miR-181a is of particular interest as it regulates type IIA activin receptor, an inhibitor of skeletal muscle proliferation through Smad 2/3 (159). However, no bioinformatics analysis was conducted on these miRNA, thus the functional implication of ameliorated age-induced altered miRNA with calorie restriction is unclear.

Interestingly, although calorie restriction returned these 5 miRNA to levels similar to younger animals, age-induced elevations in expression of miR-409, miR-223, miR-200c, miR129-5p, and miR-34a were further exacerbated with calorie restriction. Outcomes from this investigation show that while calorie restriction reversed the level of specific miRNA with aging, it may also propagate further age-induced dysregulation of miRNA within skeletal muscle. Further investigation is warranted to determine the effects of these alterations in miRNA expression in skeletal muscle. Specifically, identifying the pathways that altered miRNA profiles with calorie restriction would reveal potential harm versus benefit to skeletal muscle mass and function, especially in aging models.

Obesity (BMI > 30 $kg \cdot m^2$) in humans results in a dysregulation of c-miRNA, with Taqman® arrays covering 754 miRNA revealed that 44 miRNA were differently expressed compared to normal weight (BMI 20-25 $kg \cdot m^2$) controls (160). Following surgery-induced (bariatric surgery) weight loss, where morbidly obese (BMI > 40 $kg \cdot m^2$) patients lost 30% of initial body mass 1 year post surgery, expression of 14 c-miRNA were altered. Marked deceases were observed for miR-140-5p, miR-122, miR-193a-5p, while miR-16, while miR-221 and miR-199a-3p were upregulated in circulation. Though miR-221 and miR-199a-3p are known to interact with signaling proteins within the mTORC1 pathway, no bioinformatics analysis was conducted with this dataset, so it is difficult to interpret the functional implications of these altered expressions of c-miRNA with weight loss. Additionally, no measurement of change in skeletal muscle mass or classic assessment of protein balance (e.g., nitrogen balance, whole-body protein turnover) was conducted with surgery-induced weight loss, so whether these alterations

in c-miRNA are reflective of changes in skeletal muscle or protein balance cannot be determined.

Data from these investigations have established that calorie restriction can influence skeletal muscle and circulating miRNA expression. However, much remains unknown regarding the functional implication of altered miRNA profiles with calorie restriction. Identifying potential target pathways using bioinformatics analysis, and assessing these pathways with RT-qPCR and Western blotting techniques will be critical in future investigations. Additionally, assessment of miRNA expression in circulation should be performed concurrently with measurements of miRNA in skeletal muscle to gain a greater understanding of how expression of miRNA in circulation is associated with miRNA expression at the tissue level. Determination of c-miRNA compared to classic measurements of protein balance, such as nitrogen balance and isotope methodologies for whole-body and skeletal muscle protein turnover, would also allow for a better understanding of how to interpret the potential impact of altered skeletal muscle and circulating miRNA have on physiological adaptions in skeletal muscle following calorie restriction.

**i. Effects of dietary protein on minimizing reductions in skeletal muscle mass with calorie restriction**

Presently, appropriate interventions for the treatment of overweight and obese elderly population are highly contentious due to a lack of sufficient data demonstrating the net benefit or harm, particularly when attempting to determine the mechanism associated with attenuating losses of skeletal muscle mass (161). This has led many in the nutrition

community to investigate interventions that optimize weight loss regimens by minimizing skeletal muscle loss.

Several investigations have sought to minimize losses of muscle mass by increasing daily dietary protein intake above the current recommended dietary allowance (RDA; 0.8 $g \cdot kg^{-1} \cdot d^{-1}$) (162-164). Consumption of dietary protein at 2 times the RDA (1.6 $g \cdot kg^{-1} \cdot d^{-1}$) mitigates losses in muscle mass, with participants consuming 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ losing ~45% less muscle mass compared to consuming 0.8 $g \cdot kg^{-1} \cdot d^{-1}$ (164). Maintenance of skeletal muscle mass with provision of increased exogenous amino acids is the result of preservation in anabolic sensitivity (164), as consumption of dietary protein results in a transient elevation in the rate of skeletal muscle protein synthesis (MPS) (165).

Elevations in MPS following dietary protein intake is a dose-dependent and saturable process, with maximal stimulation being achieved with ingestion of 10 g of EAA, 20 g of an isolated high-quality protein source, or 30 g of protein as part of a mixed meal (11, 166-169). The rise in MPS in response to exogenous amino acids is rapid, reaching maximal stimulation between 60-90 minutes and returning to postabsorptive values within 180 minutes (170, 171). This stimulation of MPS to dietary protein is primarily driven by exogenous essential amino acids (EAA) (172, 173), especially the branched-chain amino acid (BCAA) leucine (174), with little contribution from non-essential amino acids (NEAA) (175). Consumption of exogenous EAA increases extracellular and intracellular concentration of EAA (169), with increased availability of EAA in muscle upregulating mTORC1 signaling (79). Activation of mTORC1 signaling by EAA is initiated by upregulation of amino acid transports, resulting in elevated intracellular concentrations. Amino acid transporters classified as solute-linked carrier (SLC) family

members are ubiquitously expressed in the plasma membrane of many cells, and typically coupled with counter-transporters, such as $Na^+/K^+$ ATPase, to maintain the ion concentration gradient (176-178). Much of the research on amino acid transporters has focused specifically on L-type amino acid transport (LAT1), which couples with a glycoprotein (CD98) and sodium-coupled neutral amino acid transporter (SNAT2). These transporters work cooperatively, with SNAT2 mediating the uptake of small neutral amino acids, in particular glutamine, which enables the bitransporter LAT1/CD98 to export glutamine to increase the influx of leucine (179). Increased expression of LAT1/CD98 and SNAT2 has been positively correlated with mTORC1 activation (180, 181). Following consumption of 10 g of EAA, LAT1/CD98 and SNAT2 expression is significantly elevated, with rates of MPS more than doubling fasting conditions (182).

Within the cell, EAA act on several signaling proteins upstream of mTORC1 to induce elevations in MPS (183). Rag GTPases, a family of four Ras-related small guanosine triphosphatases (RagA, RagB, RagC, RagD), mediate EAA induced mTORC1 activation through formation of a heterodimer which binds with the mTORC1 associated protein Raptor and triggers relocation of mTORC1 to the lysosome (184-186). Activation of the Rag heterodimeric complex is facilitated by the intracellular amino acid sensor leucyl-tRNA synthetase (LARS) directly binding to the Rag GTPase complex (187). Furthermore, amino acid induced activation of mTORC1 is facilitated by stimulation of lysosome associated vacuolar $H^+$-ATPase (v-ATPase) which controls binding of Ragulator to the Rag GTPase complex (188). Ragulator is a scaffolding complex that tethers the Rag GTPase heterodimer to the lysosome, as well as activates the complex

enabling mTORC1 binding (189). Relocation of mTORC1 increases its proximity with its activator Rheb to promote signal transduction (185).

During short-term calorie restriction there is a downregulation in both the rate of fasting and fed MPS (190-193). Recent investigations have highlighted that the amount, source, and distribution of dietary protein intake can impact MPS rates, resulting in the maintenance of skeletal muscle mass with high protein calorie restricted diets. Following 5 days of a ~30 % calorie restriction consumption of 15 g and 30 g dietary protein elevated MPS rates in a dose-dependent manner, with MPS rising 16 and 34%, respectively, compared to fasting energy balance values (190). Beyond total amount, distribution of daily protein intake can also impact the rate of MPS during periods of calorie restriction. During a 14-d 300 $kcal \cdot d^{-1}$ deficit, when participants consumed a balanced protein diet (25 g per meal) compared to a skewed protein diet (10 g breakfast, 15 g lunch, and 50 g dinner), rate of fed MPS were 19% higher for the day in the balanced versus skewed protein diets (191). Source of dietary protein intake may also contribute to the maintenance of MPS rates during periods of energy restriction. Compared to a 26 g soy protein supplementation, consumption of an equivalent amount of a whey protein supplement resulted in a > 50% rise in MPS following a 14-d 750 $kcal \cdot d^{-1}$ calorie restriction (193). Furthermore, compared to altered rates of fed MPS during energy balance, consuming a whey protein supplement only resulted in a 9% decline in MPS, while MPS decreased 25% when participants consumed a soy protein supplement. These data indicate that consuming a high quality source of dietary protein source not only results in a greater elevation in MPS, but also aids in maintaining the anabolic sensitivity of skeletal muscle. Together these investigations provide strong

evidence that the amount, daily distribution, and source of dietary protein can attenuate declines in MPS during calorie restriction and promote skeletal muscle preservation.

The underlying mechanism by which dietary protein mitigates declines in MPS and muscle mass during calorie restriction remains unclear. As dietary protein independently upregulates mTORC1 signaling, it is believed this is the likely mechanism of action, however limited evidence exist to support this claim. To date the majority of investigations assessing the role of dietary protein on anabolic signaling have been conducted during short-term (< 21 days) periods of calorie restriction. It is possible that a more sustained period of calorie restriction may be required to see a mechanistic effect of high protein diets. As such, investigation into the influence of prolonged calorie restriction with adequate or high protein diets is warranted to determine the mechanism by which dietary protein attenuates losses in muscle mass.

**j. Dietary protein and microRNA expression**

Modified expression of miRNA through the use high protein diets may be a novel intervention that aids in the maintenance of skeletal muscle mass following calorie restriction. Acute dietary protein intake has been shown to independently influence skeletal muscle miRNA expression (194). Following consumption of 10 g EAA expression of miR-1, miR-23a, miR-208b and miR-499 has been reported to be upregulated (195). With increased expression of these miRNA, target growth-related genes experience modified expression. Specifically, hypertrophic promoting genes, MyoD and FSTL1, were upregulated following consumption of EAA, while expression of myostatin, a gene that inhibits muscle growth, was downregulated. Acute modifications in miRNA and growth-related gene expression in response in ingestion of

EAA suggest that alterations miRNA expression may regulate muscle hypertrophy. Whether sustained high protein diets persistently alter miRNA expression profiles to promote increased skeletal muscle mass remains unknown.

Beyond the independent effects of dietary protein, consuming high-quality dietary protein following an acute bout of exercise results in a greater alteration to the expression of skeletal muscle miRNA. Camera *et al.* (196), reported that consumption of 25 g whey protein following concurrent exercise (leg extension 80% 1RM and 30 min cycle ergometry 70% $VO_{2peak}$) resulted in an increased expression of miR-9-3p, miR-23a-3p, miR-23b-3p, miR-133b, miR-181-5p, and miR 378-5p compared to placebo. Additionally, protein intake attenuated declines in expression of miR-494-3p. Predictive targets of these miRNA were determined using bioinformatics analysis. Assessment of predictive protein targets was performed using Western blotting. Despite having been identified as potential targets of these miRNA, there was no change in total protein content of FOXO3a, GSK-3ß, HDAC4, NRF-1, and SIRT1. Though no effect was observed on total protein, it is important to note that these assessments were conducted 4-hr after conclusion of exercise and protein consumption. It is unlikely that total protein content would be altered in this relatively short time frame. It is possible that differences would have been observed had the investigators measured mRNA expression rather than total protein content of these miRNA targets. Conversely, had investigators had participants engage in sustained concurrent training with or without post-exercise protein, differences in total protein content may have been observed. Regardless, this investigation provides evidence that skeletal muscle miRNA expression is sensitive to dietary protein intake post-exercise.

Although limited studies have been conducted, results from previous work indicates that both dietary protein and calorie restriction influence the expression miRNA within skeletal muscle. While altered miRNA profiles following acute protein consumption have been linked to muscle-growth related genes, it remains unclear if modifications in miRNA expression are a mechanism regulating alterations in skeletal muscle mass following sustained dietary interventions. Specifically, it is undefined whether sustained calorie restriction and high protein diets alter expression miRNA, and if this might be a potential mechanism regulating modification skeletal muscle mass with prolonged dietary interventions. Further investigation into the role of miRNA on alterations in anabolic molecular signaling pathways during periods of diet-induced muscle hypertrophy and/or atrophy is necessary to determine potential mechanistic functions.

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## IV. ARTICLES

### Article 1: Circulating microRNA are predictive of aging and acute adaptive response to resistance exercise in men

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Short Title: Circulating microRNA and Aging

**ABSTRACT**

Circulating microRNA (c-miRNA) have the potential to function as novel noninvasive markers of the underlying physiological state of skeletal muscle. This investigation sought to determine the influence of aging on c-miRNA expression at rest and following resistance exercise in male volunteers (Young: 22 ± 1 yrs, *n* = 9; Older: 74 ± 2 yrs, *n* = 9). Primary findings were that fasting c-miRNA expression profiles were significantly predictive of aging, with miR-19b-3p, miR-206 and miR-486 distinguishing between age groups. Following resistance exercise, principal component analysis revealed a divergent response in expression of 10 c-miRNA, where expression profiles were upregulated in younger and downregulated in older participants. Using Ingenuity Pathway Analysis to test c-miRNA-to-mRNA interactions in skeletal muscle, it was found that response of c-miRNA to exercise was indicative of an anabolic response in younger but not older participants. These findings were corroborated with a positive association observed with the phosphorylation status of p-Akt$^{Ser473}$ and p-S6K1$^{Thr389}$ and expression of miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, and miR-195-5p. These important findings provide compelling evidence that dysregulation of c-miRNA expression with aging may not only serve as a predictive marker, but also reflect underlying molecular mechanisms resulting in age-associated declines in skeletal muscle mass, increased fat mass, and 'anabolic resistance'.

**Key Words**: Anabolic resistance, miR-19b-3p, miR-206, miR-486

## INTRODUCTION

Deleterious changes in body composition, where there is a concomitant loss of skeletal muscle mass and increase in fat mass, is a consistent pathophysiological condition that occurs with aging (1, 2). Without intervention, these detrimental modifications in body composition increase the risk of developing metabolic syndrome and diminished mobility and physical function, ultimately decreasing health-span and elevating healthcare costs (3, 4). Age associated alterations in body composition are not only attributed to reduced physical activity, but also a blunting of cellular mechanisms involved in the regulation of muscle hypertrophy in response to an acute stimulus, termed 'anabolic resistance' (5, 6). Recently, our laboratory (7) identified altered expression of microRNA (miRNA; small non-coding RNA that negatively regulate gene expression (8)) in skeletal muscle as a mechanism for impairment of exercise-induced adaptations in older individuals. Specifically, discordant response of miRNA expression to a bout of resistance exercise with aging inhibited the acute adaptive response of insulin-like growth factor 1 (IGF-1) signaling (7). Similarly, altered expression of miRNA with aging has been observed to predict the anabolic response of the downstream target of IGF-1, mechanistic target of rapamycin complex 1 (mTORC1), which is the central regulator of hypertrophy and muscle mass (9).

Though assessment of miRNA expression in skeletal muscle has yielded valuable insight into their functional role with age-induced changes to body composition, this type of data collection requires invasive muscle biopsies, which may cause discomfort, and limit tissue available for analysis. Determination of noninvasive markers is thus warranted to capture larger datasets to improve the understanding of mechanisms altering

body composition and metabolic health with aging. Recently, miRNA have been reported to be present in circulation (c-miRNA), with alterations in c-miRNA profile reflective of the underlying physiological state that negatively impact skeletal muscle mass (10-12). The ability of c-miRNA expression to provide insight into changes of pathogenic processes in skeletal muscle makes them a promising noninvasive marker. While aging has been demonstrated to alter c-miRNA profiles (13-16), whether these differences relate to age-associated alterations in body composition and metabolic health have not been assessed. Furthermore, it remains undetermined if the expression of c-miRNA following an acute anabolic stimulus can distinguish 'anabolic resistance' in older individuals.

The objective of the present investigation was to assess the influence of aging on c-miRNA expression under resting fasted conditions, and determine their relationship to body composition and circulating metabolic markers of health. Furthermore, this investigation sought to determine the influence of an acute bout of resistance exercise on changes in c-miRNA expression, and establish if c-miRNA are predictive of exercise-induced adaptions. We hypothesized that aging would result in altered c-miRNA profiles associated with differences in body composition and circulating metabolic markers of health. Additionally, we hypothesized that following resistance exercise a divergent response in c-miRNA expression with aging will be predictive of 'anabolic resistance.' Overall, findings from this investigation demonstrate the predictive and potentially mechanistic role of c-miRNA in age-associated decrements in body composition.

## METHODS

### Participants and study design

Data for this analysis were collected as part of an investigation aimed to assess markers of anabolism and catabolism in younger and older individuals in response to an acute bout of exercise (7, 17). For the present investigation data were analyzed on a total of 18 younger (22 ± 1 yrs, *n* = 9) and older (74 ± 2 yrs, *n* = 9) male participants (**Supplemental Table 1**). As previously described, all participants were in good health as determined by medical screening and did not regularly engage in resistance or endurance exercise. Data collection was performed at the United States Department of Agriculture (USDA) Human Nutrition Research Center on Aging (HNRCA). This investigation was approved by the Tufts University Health Sciences Campus Institutional Review Board, with informed written consent obtained from all participants.

One week prior to data collection, one repetition maximum (RM) was determined for all participants to prescribe exercise intensity for the acute resistance exercise bout. Participants were admitted to the HNRCA 24-hr prior to the acute resistance exercise bout. Baseline blood samples were collected at this time. The following morning participants performed 3 sets of bilateral knee extension exercise and 3 sets of bilateral leg press exercise for 10 repetitions at 80% of their 1 RM. Blood sampling was conducted immediately (0-hr) post exercise and again at 6-hrs post exercise (recovery). Participants were fasted throughout the entire 6-hr data collection period.

**Anthropometrics and Body Composition**

Height was measured to the nearest 0.1 cm using a wall mounted stadiometer. Body mass and composition were determined using dual-energy x-ray absorptiometry (DXA; Hologic, Bedford, MA) as previously described (17).

**Blood Sampling**

Blood samples were obtained at baseline, post exercise, and recovery. All samples were obtained following an overnight fast. To minimize hemolysis and potential contamination of serum by red blood cells, venipuncture was performed by a registered nurse trained in phlebotomy at each time point of the trial. Blood was allowed to clot at room temperature and then centrifuged at 2,135g for 10 minutes at 4°C. Derived serum were stored in 500 µL aliquots at −80°C prior to analysis. No serum sample that was visual hemolyzed (red in color) was used in analysis.

**Substrate and Hormone Analysis**

Serum glucose and triglyceride concentrations were assessed using Beckman Coulter AU400e Chemistry analyzer (Beckman Coulter, Inc., Brea CA).

**Circulating microRNA Extraction and Expression**

Circulating miRNA were extracted from 200 µL serum using miRNeasy Serum/Plasma kit, which allows for extraction and purification of small (< 200 nt) cell-free RNA (217184; Qiagen Valencia, CA). To avoid introducing potential contaminating material, prior to RNA extraction serum samples were centrifuged for 10 min at 4°C to remove cellular debris. Supernatant was removed and transferred to a new tube without disturbing the pellet. Due to the small amount of RNA in the serum, 3.5 µL of a Spike-In Control (C. elegans miR-39; 219610; QIAGEN) was added to all samples prior to extraction of RNA to determine the yield of template recovered. Reverse transcription (RT) using a fixed amount (1.5 µL) of RNA was performed using miScript II RT Kit (218161; QIAGEN). Real-time polymerase chain reaction (RT-qPCR) amplifications were conducted following manufactures instructions using a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA) to assess cDNA template in a

384 well miScript miRNA PCR Array (MIHS-3106Z; QIAGEN). Arrays were run using a miScript SYBR Green PCR Kit, quantifying 84 miRNA.

Additional miRNA of interest (miR-34a, miR-181, miR-206, miR-208b, miR-324, miR-486) were analyzed using TaqMan® MicroRNA Assays (4427975;Applied Biosystems) following previously described multiplex RT and pre-amplification protocol (18). Briefly, miRNA were reverse-transcribed using the TaqMan® microRNA RT kit (4366596; Applied Biosystems) with the eight miRNA-specific stem-loop RT primers pooled in 1X-Tris-EDTA (TE) buffer for a final dilution of 0.05X for each miRNA RT primer. The RT primer pool (6 µl) was added to the RT reaction mix (0.3 µl 100mM dNTP, 3 µl enzyme, 1.5 µl 10× RT buffer, 0.19 µl RNase inhibitor) and 3 µl of serum RNA. A pre-amplification step was performed to increase cDNA template using a primer pool of 20 X Taqman® Small RNA Assay for the eight miRNA of interest at 0.05X concentration in 1X TE buffer. Pre-amplification reaction mix was constituted of 3.75 µl primer pool, 2.5 µl cDNA, 12.5 µl Taqman® Universal PCR Master Mix (2X), no UNG (#4440040 Applied Biosystems) and 6.25 µl nuclease free $H_2O$. Reverse transcription and pre-amplification were conducted following manufactures instructions in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). Following pre-amplification, RT-qPCR amplifications were conducted following manufactures instructions using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

If a miRNA did not cross the cycle threshold ($C_t$) for any time point or if the average miRNA expression was $\geq 35$ $C_t$ (19) it was not included in analysis (**Supplemental Table 2**). All miRNA were normalized to the geometric mean of Spike-In Control miR-39 (external control) and SNORD 95 and U6 (internal controls). A total of 6 internal

controls (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and U6) were assessed for this analysis. SNORD 95 and U6 were determined to be the most stable and least variable internal controls in the present investigation. Combination of external and internal controls allows for both technical and inter-individual normalization (20). Geometric mean of controls was used to correct for possible outlying values and abundance differences between the different controls (21). Furthermore, geometric mean of Spike-In Control miR-39, SNORD 95 and U6 was determined to be a homogenously and stably expressed housekeeper with a coefficients of variation (CV) of 4.5% (22). Fold changes were calculated using the ΔΔ cycle threshold ($\Delta\Delta C_T$) method as described below in statistical analysis section.

**Discriminate Analysis**

To determine a potential grouping of c-miRNA that distinguishes between age groups, stepwise discriminant analysis with forward selection based on Wilk's lambda was conducted. A non-significant result for Box's test of equality of covariance matrices confirmed that the model was appropriate for discriminant analysis. A cross-validation method was performed to assess the reliability and generalizability for results from discriminant analysis. Based on outcomes from discriminant analysis, receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to assess the potential for miR-19b-3p, miR-206, miR-221-3p, and miR-486 as predictive markers for aging.

**Clustering and Assessment of miRNA Associated Pathways**

Principal component analysis (PCA) was performed on miRNA with a fold difference of +/- 1.5 between young and older at baseline or fold change of +/- 1.5 at post exercise or

recovery compared to baseline. This analysis allowed for grouping of relevant miRNA, while controlling for type 1 error. The dataset was verified suitable for factor analysis by a Kaiser-Meyer-Olkin of Sampling Adequacy > 0.6 with a significant < 0.05 Bartlett's Test of Sphericity. Related clusters of miRNA were then uploaded to DNA Intelligent Analysis (DIANA)-mirPath 3.0 (Alexander Fleming Biological Sciences Research Center (BSRC), Athens, Greece; http://diana.cslab.ece.ntua.gr) to determine potential molecular pathways that these miRNA have previously been reported to regulate. Relevant Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www. genome.jp/kegg/) pathways were identified using experimentally verified targets from TarBase 7.0 (Alexander Fleming BSRC).

**Ingenuity Pathway Analysis**

Ingenuity miRNA Target Filter analysis was conducted for all possible c-miRNA-to-mRNA interactions from the top 10 c-miRNA identified in Component 1 of PCA results to gene array dataset of altered mRNA expression in skeletal muscle 6-hr post resistance exercise in young and older (7). Data of differentially expressed c-miRNA and mRNA were integrated using the expression pairing function to obtain c-miRNA-to-mRNA relationships. The interacting networks were further explored for relevance to biological pathways and upstream regulators. Experimentally validated interactions and predictions characterized as "high" confidence were considered based on TargetScan and Ingenuity Knowledgebase. Based on top canonical pathways observed in younger and older participants the association of the top 10 c-miRNA identified in Component 1 of PCA results to phosphorylation status of p-Akt$^{Ser473}$ and p-S6K1$^{Thr389}$ were determined from

data obtained from a previous investigation of these subjects (7). Phosphorylation of these protein targets were determined with Western blotting as previously published (7).

**Statistical Analysis**

Normality of data was assessed using Shapiro–Wilk tests for dependent variables. Data were not normally distributed ($P < 0.05$) and fold change data for c-miRNA were log transformed ($\log_2$) for statistical analysis, but presented as original values (mean ± SEM). Baseline data (younger and older) were set as control to calculate fold change ($2^{-\Delta\Delta CT}$) of c-miRNA expression at baseline, post exercise, and recovery. Mixed-model repeated measures ANOVA were performed to determine main effects of age (young and older), time (baseline and post exercise and recovery) and age-by-time interaction. Based on Akaike's information criterion, unstructured covariance was determined as the appropriate model for this analysis. For c-miRNA with a significant main effect of time or age-by-time interaction, Bonferroni correction was used for *post hoc* pairwise comparisons. Backwards linear regression analysis was conducted to determine the relationship of miR-19b-3p, miR-206 and miR-486 to age, fat-free mass, fat mass, and glucose and triglyceride concentrations. Spearman's rho rank correlation coefficient was utilized to determine associations. The α level for significance was set at $P \leq 0.05$. Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp. Armonk, NY).

**RESULTS**

**Expression of miR-19b-3p, miR-206 and miR-486 Discriminant of Aging**

Of the 90 miRNA assessed, 65 miRNA crossed the cycle threshold ($C_t$) before 35 cycles in both younger and older participants at each time point (**Supplemental Table 2**).

Stepwise discriminant analysis revealed four c-miRNA (miR-19b-3p, miR-206, miR-221-3p, miR-486) that correctly classified 93% of participants by age, with cross-validated group cases correctly classified with 89% accuracy (**Figure 1A)**. Receiver operating characteristic (ROC) curve analysis was then conducted, measuring the sensitivity (true positive) and specificity (false positive) of these four c-miRNA. miR-19b-3p differentiated aging with an AUC of 0.75, sensitivity 89% and specificity 67% (**Figure 1B**). miR-206 distinguish aging with an AUC of 0.70, sensitivity 89% and specificity 78%, while miR-486 had an AUC of 0.77, sensitivity 89% and specificity 89%. Though miR-221-3p was identified in the stepwise discriminant analysis, it was not sensitive or specific to age using ROC curve analysis.

Accordingly, backwards linear regression was conducted to test the association of miR-19b-3p, miR-206 and miR-486 expression to descriptive characteristics, which included age, fat-free mass, fat mass, and glucose and triglyceride concentrations (**Supplemental Table 1**). Positive and negative associations of fat-free mass and glucose concentrations, respectively, accounted for 36% of the variance in miR-19b-3p expression ($r = 0.60$, $r^2 = 0.36$; $P = 0.04$), triglyceride concentrations accounting for 47% of the variance ($r = 0.68$, $r^2 = 0.47$; $P < 0.01$) in miR-206 expression, and fat mass and glucose concentration explained 52% of the variance ($r = 0.72$, $r^2 = 0.52$; $P < 0.01$) for miR-486 expression. Furthermore, when fat-free mass and fat mass were expressed as a percentage of total body weight to account for difference in body weight between age groups, percent fat-free mass was negatively associated with miR-206 ($r = -0.51$, $r^2 = -0.16$; $P = 0.03$) and miR-486 ($r = -0.65$, $r^2 = -0.39$; $P < 0.01$), while percent fat mass was

positively associated with miR-206 ($r = 0.56$, $r^2 = 0.22$; $P = 0.02$) and miR-486 ($r = 0.68$, $r^2 = 0.39$; $P < 0.01$).

To determine if common regulatory function could explain the association of discriminate miRNA to body composition and markers of metabolic health KEGG pathways were identified using predicted targets of miR-19b-3p, miR-206 and miR-486 using DIANA-miRPath 3.0. Mutual relevant pathways were identified as FoxO signaling, p53 signaling, cell cycling, PI3K-Akt signaling, apoptosis, and AMPK signaling, revealing that these 3 distinguishing miRNA serve as regulators of skeletal muscle anabolism and atrophy, metabolism, immune-regulation, and oxidative stress. All KEGG pathways common to of miR-19b-3p, miR-206 and miR-486 are presented in **Supplemental Table 3**.

Altogether, these comprehensive analysis not only identified miR-19b-3p, miR-206 and miR-486 as predictive of aging, but common molecular pathways and associations with body composition and markers of metabolic health plausibly illustrate their critical role as regulators in aging processes.

**Circulating miRNA Predictive of 'Anabolic Resistance' with Aging**

Impairment of the acute adaptive response to exercise in skeletal muscle with aging (e.g., anabolic resistance) plays an important role in age-associated alterations in body composition (6). Though determination of molecular adaptions in muscle typically require invasive muscle biopsies procedures, assessment of c-miRNA expression may provide insight into the underlying physiological state of tissue while minimizing discomfort the participant. In the current investigation aging and/or response to acute resistance exercise altered ($P < 0.05$) the expression of 25 c-miRNA (**Supplemental**

**Table 4; Figure 2A**). Principal Component Analysis (PCA) was performed to group c-miRNA based on fold change in response to aging and exercise. Five c-miRNA components were extracted from PCA with Eigenvalues > 1. Of these 5 c-miRNA components, Component 1 explained the majority of the variance (60%) in the dataset (**Supplemental Table 5**), while the remaining 4 components combined only explained 24% of the variance in the dataset. Additional analysis was performed on the top 10 c-miRNA in Component 1 to assess between group differences and associated pathways. A main effect of age ($P < 0.05$) was observed for 9 of the 10 c-miRNA, with c-miRNA expression begin greater in young compared to older participants (**Figure 2B**). To determine potential regulatory function, these 10 c-miRNA were uploaded into DIANA-miRPath 3.0, with KEGG pathways identified using predicted targets. Relevant pathways related to exercise-induced adaptations containing at least 8 of the10 c-miRNA were reported (**Figure 3A**). Disease related targets, such as cancers, were not reported. Results of this analysis revealed that these 10 c-miRNA predicted hypertrophic, proteolytic, inflammatory, and metabolic pathways whose activity have characterized to be augmented with exercise.

To further characterize the potential use of c-miRNA as a marker of aging and acute adaptive response to exercise, Ingenuity Pathway Analysis (IPA) was on c-miRNA in Component 1 of PCA to skeletal muscle mRNA at 6-hr into recovery of resistance exercise (7). Results of IPA showed that top regulated canonical pathways differed with aging, as anabolic pathways, IGF-1 and mTOR signaling, were present in younger, but not older participants (**Table 1**). Furthermore, the top physiology system development and function category produced by IPA for younger participants was skeletal and

muscular system development, which again was not present in the results for older participants. Absence of anabolic signaling in the IPA results with aging suggests that a downregulation of the 10 c-miRNA in Component 1 is indicative of 'anabolic resistance'. Strengthening the IPA results, positive correlations were observed between the expression of c-miRNA in Component 1 compared to phosphorylation status of up and downstream targets of mTORC1, p-Akt$^{Ser473}$ and p-S6K1$^{Thr389}$. Of the 10 c-miRNA in Component 1, miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, and miR-195-5p were significantly associated with both p-Akt$^{Ser473}$ and p-S6K1$^{Thr389}$ (**Figure 3B-C, Supplemental Table 6**). These findings highlight that not only is the expression of c-miRNA altered following exercise, but that this change may be reflective of the underlying molecular processes being activated in skeletal muscle.

**DISCUSSION**

The main findings from the present investigation provide initial evidence that c-miRNA have the potential to function as noninvasive markers of physiological and metabolic adaptations with aging and acute adaptive response to resistance exercise. Using advanced statistical methodologies and integrative bioinformatics analysis this investigation is the first to report that alterations in c-miRNA expression profiles with aging are predictive of age-associated modifications in body composition and metabolic health. Furthermore, combining measurements of c-miRNA expression with skeletal muscle mRNA and signaling proteins, findings from this investigation not only show that aging results in a divergent c-miRNA profile following resistance exercise, but that this discordant response may be reflective of underlying acute adaptive responses in skeletal muscle.

Paralleled reductions in skeletal muscle mass and increased fat mass with aging results in impaired whole-body metabolism, over time propagating a feed-forward loop leading to further reductions in muscle and elevated fat mass (23), a process termed 'sarcopenic obesity.' Due to this concomitant shifts in body composition, metabolically compromised older individuals may maintain a normal BMI (< 25 $kg \cdot m^{-2}$), masking their risk for developing weight related comorbidities such as cardiovascular disease and diabetes (24). In the present investigation, despite having a BMI only slightly above normal (25.6 $kg \cdot m^{-2}$), we observed that c-miRNA (miR-19b-3p, miR-206 and miR-486) which distinguished aging were associated with body composition and markers of metabolic health (e.g., serum glucose and triglyceride concentrations). The ability for these c-miRNA to discriminate aging, despite older participants being of good health, suggests c-miRNA expression may be a highly sensitive predictive tool for development of more severe conditions, such as sarcopenic obesity and metabolic syndrome. Previous investigations have provided promising evidence that c-miRNA expression is reflective of metabolic health. Morbidly obese (BMI > 40 $kg \cdot m^{-2}$) and diabetic participants experience similar alterations in c-miRNA expression profiles, with treatment (e.g., bariatric surgery and Metformin) improving c-miRNA expression profiles such that data are comparable to healthy control participants (12, 25). Additionally, c-miRNA have been shown to be predictive of improvements in insulin resistance, with circulating miR-486 expression indicative of responders and non-responders to Thiazolidinedione therapy (26). Given that potential functions of discriminant miRNA in the present investigation may regulate pathways involved in skeletal muscle anabolism and atrophy, metabolism, immune-regulation, and oxidative stress (27-30), dysregulation of their expression in

circulation may be attributed to the aging process. While findings from the present investigation cannot conclusively demonstrate that dysregulation in these discriminate miRNA alter skeletal muscle physiology with aging, these results do provide compelling preliminary evidence to warrant further in-depth investigation.

Assessment of the acute response to an anabolic stimulus in aging is critical when assessing mechanism related to age-associated alterations in body composition, as a blunted response (e.g., 'anabolic resistance') to such events potentially results in declines in skeletal muscle mass and increased fat mass (31, 32). While several studies have investigated altered expression of c-miRNA in response to an acute bout of aerobic exercise (33-35), the response of c-miRNA expression to an acute bout of resistance exercise with aging remains undefined. Findings from the present investigation are the first to report that not only is there a divergent response in the expression of specific c-miRNA with aging, but also that up or downregulation of these c-miRNA may be indicative of underlying physiological processes in skeletal muscle following resistance exercise. Functional analysis of c-miRNA-mRNA interactions using IPA elucidated an absence of an anabolic response to exercise with aging, as the well-characterized hypertrophic signaling cascades, IGF-1 and mTOR, were identified as top canonical pathways in young, but not aged males. This finding is consistent with the hypothesis that aging results 'anabolic resistance' (5, 6), where the synthetic response to an acute anabolic stimulus such as resistance exercise is blunted (36-38). Further strengthening the bioinformatics data of our current investigation, a positive association was observed between the expression of c-miRNA in Component 1 of PCA to the phosphorylation status (e.g., activity) of p-Akt$^{Ser473}$ and p-S6K1$^{Thr389}$. This finding shows that an

upregulation in the expression of these c-miRNA, in particular miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, and miR-195-5p, are indicative of a hypertrophic response within skeletal muscle. Coupling state-of-the-art integrative analytic techniques with findings from traditional bench top techniques assists in validating that our IPA findings are accurate, and that c-miRNA can be used as noninvasive markers to predict adaptations reflective of molecular processes in skeletal muscle to acute resistance exercise with aging.

Interestingly, many of the c-miRNA (miR-17-3p, miR-18a, miR-19a, miR-19b, and miR-20a) observed in Component 1 of PCA results are members of the miR-17~92 cluster (39). Additionally, this cluster has been extended two paralog families of miRNA which contain miR-93 and miR-106b (40). These clusters of miRNA are generated from a primary transcript and have a large overlap in their functions (40). Though little is known regarding the influence of acute resistance exercise on this cluster of microRNA, disease models have shown convergence of these miRNA on Akt-mTOR signaling within tissue (41). A main target of these miRNA is PTEN, an inhibitor of the PI3K-Akt pathway. Inhibition of PTEN can promote cellular survival and proliferation through increased activation of Akt-mTOR signaling (39). Results from these previous investigations corroborate findings from the present study; c-miRNA in Component 1 target anabolic pathways (39-41). Furthermore, identification of divergent c-miRNA profiles following resistance exercise being members of the same family of miRNA, sharing similar target genes, further strengthens the potential use of c-miRNA as potential predictive markers of resistance exercise-induced adaptions.

Use of c-miRNA expression profiles as a predictive biomarker for the pathophysiological state of tissue has gained favor in recent years, as c-miRNA have been shown to be stable, reproducible, and predictive of clinical outcomes in disease states (42). The biogenesis and maturation of miRNA occur in the nucleus and cytoplasm of the cell, respectively (43, 44). Mature miRNA can be released into the circulation in membrane-derived vesicles (exosomes), complexed with proteins, lipoproteins (cholesterol), or apoptotic bodies (plasma membrane fragments) (45). As miRNA serve to regulate anabolic and metabolic molecular pathways, with transport of miRNA in the circulation potentially involved in cell-to-cell communication (45, 46), dysregulation of c-miRNA expression with aging may not only serve as predictive marker, but may also provided insight into underlying mechanisms resulting in age-associated declines in skeletal muscle mass, increased fat mass, and 'anabolic resistance'. While presently it can be observed how c-miRNA expression profiles are altered under various conditions, and potential function can be inferred using bioinformatics analysis, causation cannot be determined. Advancement of methodologies to isolate exosomes and microvesicles, as well as determination of tissue source *in vitro* are required to fully realize the functional potential of c-miRNA to determine causal effects (47). Future investigations should focus on attempting to isolate skeletal muscle derived miRNA in serum to gain further insight into their use as noninvasive markers of altered skeletal muscle physiology with aging.

To the best of our knowledge, results from this work are the first of their kind to identify c-miRNA as predictive markers for aging and 'anabolic resistance'. Although this investigation was limited in sample size and conducted only in male participants,

here we demonstrated that aging results in altered c-miRNA expression profiles with distinguishing miR-19b-3p, miR-206 and miR-486 being associated with body composition and metabolic health. Furthermore, using advanced statistical and bioinformatics analysis we were able show that divergent responses in c-miRNA expression were predictive of acute adaptations to resistance exercise, where c-miRNA expression was associated with altered phosphorylation status of up and downstream targets of mTORC1. Enhanced understanding of alterations in c-miRNA expression with aging may result in clinically relevant diagnostic tools and further substantiating the use of 'liquid biopsies' in aging diseases. Unlike traditional markers of health, c-miRNA are not end products of altered molecular pathways, but rather serve in a functional role. As such, deregulation of c-miRNA with aging and association to body composition and metabolic health may not only be used to predict risk of developing conditions such as sarcopenic obesity and metabolic syndrome, but also provide an understanding into mechanisms involved in the aging process.

**Conflict of Interest**

The authors report no conflicts of interest.

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**Table 1**. c-miRNA Component 1 comparison analysis to skeletal muscle gene expression

| Younger | *P* | Older | *P* |
|---|---|---|---|
| | | **Canonical Pathways** | |
| AMPK Signaling | 7.28E-05 | AMPK Signaling | 6.21E-04 |
| Coagulation System | 2.64E-04 | Xenobiotic Metabolism Signaling | 1.63E-0.3 |
| ***IGF-1 Signaling*** | 4.39E-04 | Glucocorticoid Receptor Signaling | 1.75E-03 |
| ILK Signaling | 5.70E-04 | IL-10 Signaling | 1.77E-03 |
| ***mTOR Signaling*** | 6.57E-04 | Pyridoxal 5'-phosphate Salvage Pathway | 1.87E-03 |
| | | **Physiology System Development and Function** | |
| ***Skeletal and Muscular System Development*** | 1.96E-03 – 7.27E-08 | Connective Tissue Development and Function | 2.93E-03 – 4.29E-07 |
| Tissue Development | 1.96E-03 – 9.23E-08 | Cardiovascular Development and Function | 3.57E-03 – 2.09E-06 |
| Connective Tissue Development and Function | 1.96E-03 – 1.42E-07 | Organismal Development | 3.74E-03 – 2.09E-06 |
| Tissue Morphology | 2.07E-03 – 1.42E-07 | Tissue Development | 3.48E-03 – 2.09E-06 |
| Organismal Survival | 1.55E-04 – 3.40E-07 | Tissue Morphology | 3.48E-03 – 1.07E-05 |

Functional analysis of c-miRNA-to-mRNA data interactions using Ingenuity Pathway Analysis with the miRNA target filter. Italics denote relevant pathways.

**Figure Legends**

**Figure 1.** Discriminant analysis using miR-19b-3p, miR-206, miR-221-3p, miR-486 for Young (♦) and Older (♦) under fasted resting conditions (***A***). Receiver Operating Characteristic (ROC) curve analysis to determine sensitivity and specificity of miR-19b-3p, miR-206, miR-486 (***B***).

**Figure 2.** Fold change c-miRNA expression baseline, 0-hr post exercise (PE) and 6-hr post exercise (REC) in Younger (YNG) and Older (OLD; **A**). Fold change of c-miRNA from Component 1 from PCA at baseline (■), 0-hr post exercise (■) and 6-hr post exercise (□) in Younger (YNG) and Older (OLD; **B**). Fold change calculated using average baseline data for both younger and older participants as controls. Mixed model repeated measures ANOVE with Bonferroni adjustment used to determine main effect of age, time, and age-by-time interaction. Data presented as mean ± SEM. *Main effect of age, older significantly different from younger participants; $P < 0.05$. †Main effect of time; $P < 0.05$.

**Figure 3.** Identification of significant ($P < 0.05$) and relevant KEGG pathways associated with exercise-induced adaptations and number of genes regulated by top 10 c-miRNA in Component 1 (**A**). Determined with PCA and identified with DIANA-miRPath 3.0 using experimentally verified targets from TarBase 7. Correlation of c-miRNA expression to phosphorylation status of $Akt^{Ser473}$ (**B**) and $S6K1^{Thr389}$ (**C**) from Western blot analysis conducted in a previous investigation (7). Associations determined using Spearman's Rho rank correlation coefficient. All correlations significant; $P < 0.05$.

**Figure 1.** Discriminant and ROC curve analysis of miR-19b-3p, miR-206 and miR-486

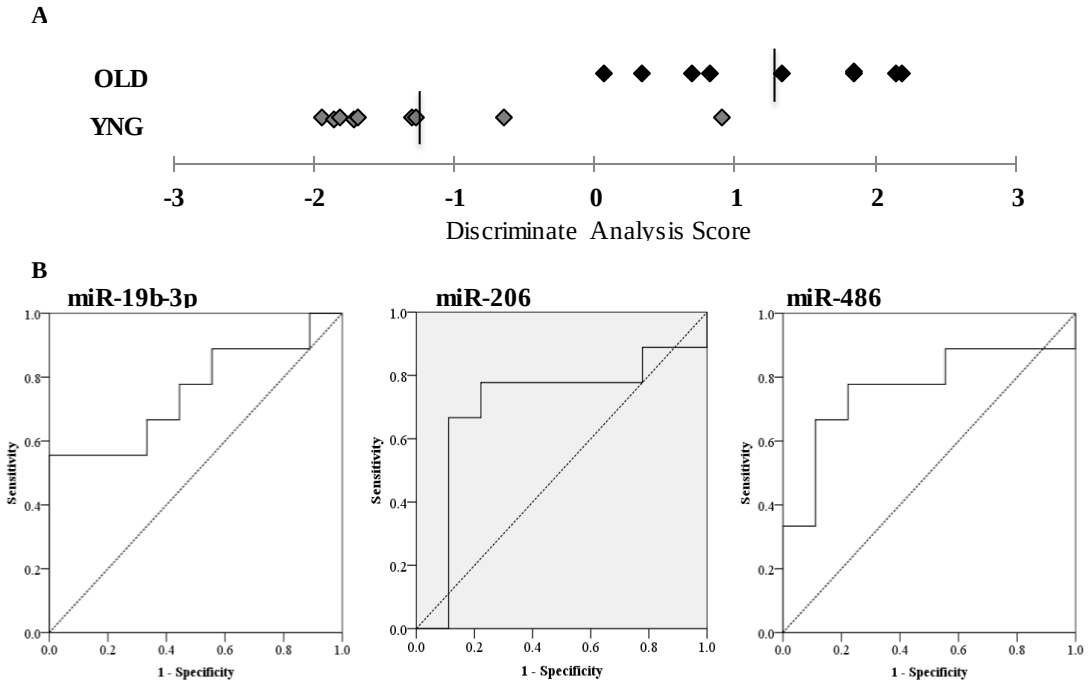


**Figure 2.** Fold change c-miRNA expression to aging and acute exercise

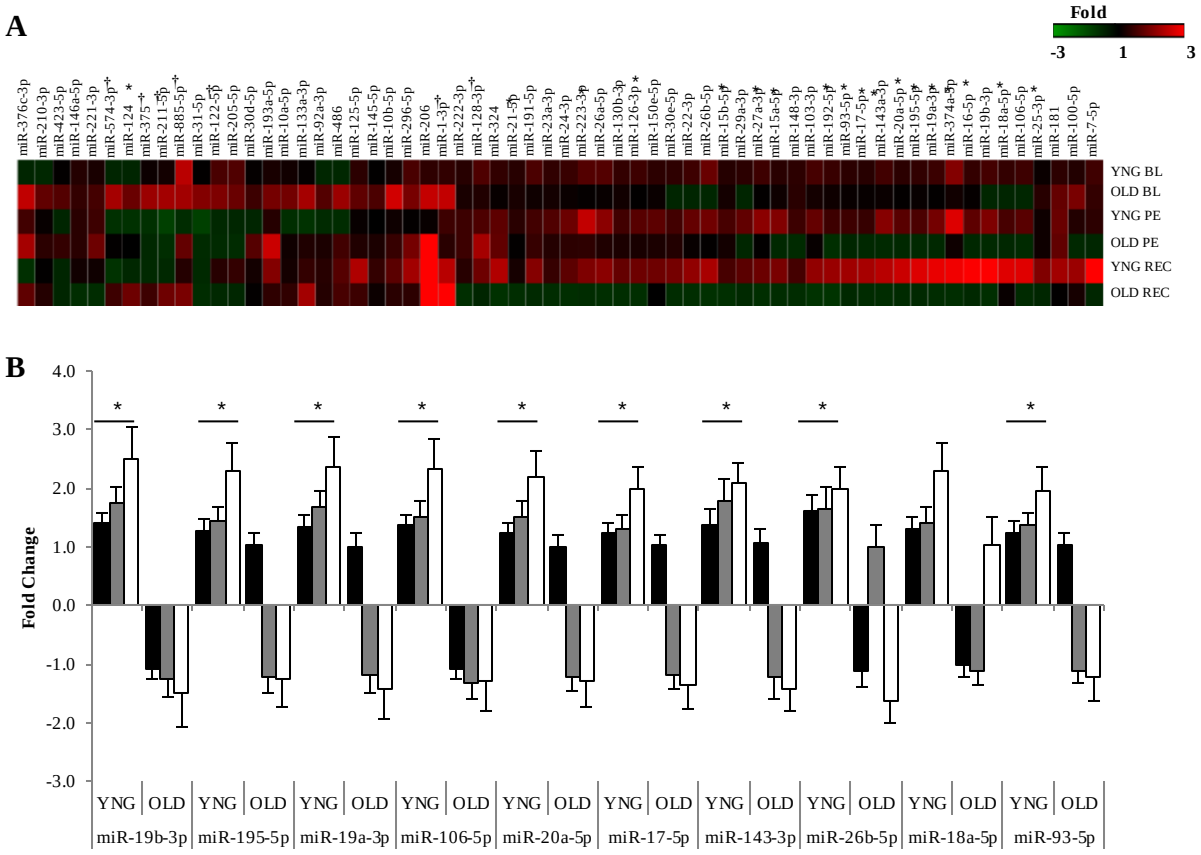
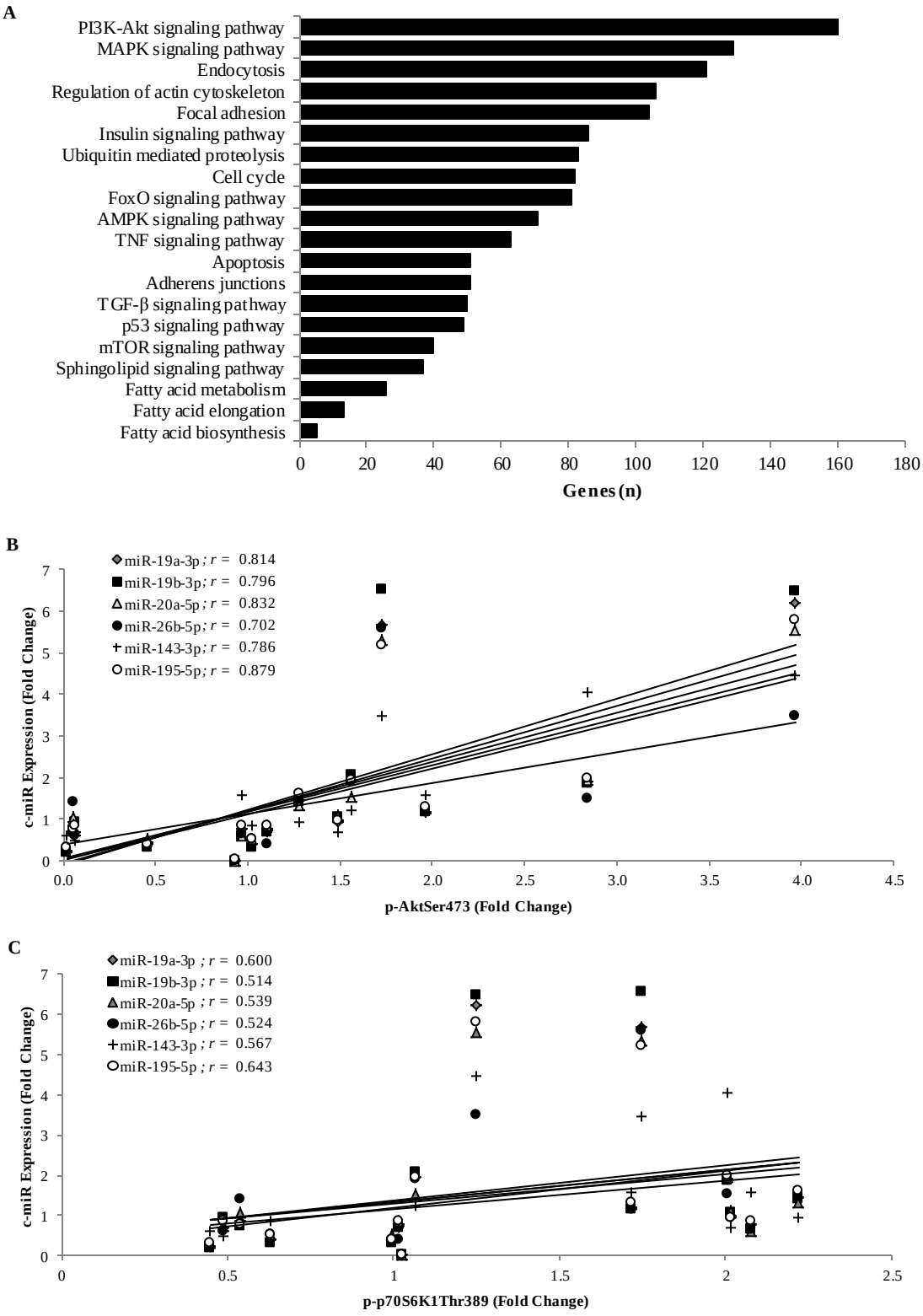


**Figure 3.** Pathway analysis PCA Component 1 c-miRNA



**Supplemental Table 1.** Participant characteristics

| | **Younger (n = 9)** | **Older (n = 9)** |
|---|---|---|
| **Age (yrs)** | 22 ± 1 | 74 ± 2* |
| **Weight (kg)** | 68.7 ± 1.9 | 77.8 ± 3.4* |
| **Height (cm)** | 178.1 ± 1.4 | 174.2 ± 1.9 |
| **BMI ($kg \cdot m^{-2}$)** | 22.1 ± 0.7 | 25.6 ± 0.8* |
| **Fat-Free Mass (kg)** | 57.0 ± 1.8 | 58.3 ± 2.6 |
| **Fat Mass (kg)** | 9.2 ± 0.9 | 16.9 ± 1.3* |
| **Glucose ($mg \cdot dL^{-1}$)** | 89.6 ± 2.0 | 95.6 ± 3.4 |
| **Triglycerides ($mg \cdot dL^{-1}$)** | 55.3 ± 6.4 | 89.7 ± 11.7* |

Mean ± SEM

Independent t-test determine between group differences

*Significantly different than Young; $P < 0.05$

**Supplemental Table 2**. Average cycle threshold (Ct) values of c-miRNA

| **Expressed Consistently** | | **Not Expressed Consistently**[1] | |
|---|---|---|---|
| **miRNA** | **Ct** | **miRNA** | **Ct** |
| let-7a-5p | 29.01 | miR-107 | 35.37 |
| let-7c-5p | 31.64 | miR-133b | 35.67 |
| miR-100-5p | 31.85 | miR-134-5p | 37.30 |
| miR-103a-3p | 29.56 | miR-141-3p | 38.06 |
| miR-106b-5p | 27.96 | miR-155-5p | 37.24 |
| miR-10a-5p | 33.93 | miR-17-3p | 36.12 |
| miR-10b-5p | 34.98 | miR-184 | 39.61 |
| miR-122-5p | 29.55 | miR-196a-5p | 37.65 |
| miR-124-3p | 26.28 | miR-200a-3p | 38.78 |
| miR-125b-5p | 31.29 | miR-200b-3p | 37.42 |
| miR-126-3p | 27.53 | miR-200c-3p | 36.44 |
| miR-128-3p | 32.53 | miR-203a-3p | 38.36 |
| miR-130b-3p | 32.76 | miR-204-5p | 38.78 |
| miR-133a-3p | 34.62 | miR-208a-3p | 39.84 |
| miR-1-3p | 33.46 | miR-214-3p | 35.95 |
| miR-143-3p | 32.24 | miR-215-5p | 38.03 |
| miR-145-5p | 30.52 | miR-224-5p | 36.14 |
| miR-146a-5p | 30.51 | miR-34a-5p | 35.23 |
| miR-148a-3p | 30.17 | miR-372-3p | 39.55 |
| miR-150-5p | 27.94 | miR-373-3p | 37.86 |
| miR-15a-5p | 31.11 | miR-499a-5p | 38.50 |
| miR-15b-5p | 28.90 | miR-9-5p | 39.01 |
| miR-16-5p | 24.60 | miR-96-5p | 38.47 |
| miR-17-5p | 28.54 | SNORD61 | 34.08 |
| miR-18a-5p | 30.47 | SNORD68 | 32.79 |
| miR-191-5p | 28.04 | SNORD72 | 36.49 |
| miR-192-5p | 32.66 | SNORD96A | 33.29 |
| miR-193a-5p | 33.86 | | |
| miR-195-5p | 25.42 | | |
| miR-19a-3p | 27.76 | | |
| miR-19b-3p | 28.54 | | |
| miR-205-5p | 32.92 | | |
| miR-20a-5p | 27.07 | | |
| miR-210-3p | 33.81 | | |
| miR-211-5p | 35.68 | | |
| miR-21-5p | 27.32 | | |
| miR-221-3p | 29.11 | | |
| miR-222-3p | 30.48 | | |
| miR-223-3p | 24.93 | | |

| | | | |
|---|---|---|---|
| miR-22-3p | 28.16 | | |
| miR-23a-3p | 27.17 | | |
| miR-24-3p | 28.49 | | |
| miR-25-3p | 27.41 | | |
| miR-26a-5p | 28.20 | | |
| miR-26b-5p | 29.00 | | |
| miR-27a-3p | 28.21 | | |
| miR-296-5p | 34.39 | | |
| miR-29a-3p | 29.32 | | |
| miR-30d-5p | 28.34 | | |
| miR-30e-5p | 28.25 | | |
| miR-31-5p | 35.59 | | |
| miR-374a-5p | 32.85 | | |
| miR-375 | 31.60 | | |
| miR-376c-3p | 34.09 | | |
| miR-423-5p | 30.18 | | |
| miR-574-3p | 30.41 | | |
| miR-7-5p | 33.44 | | |
| miR-885-5p | 33.53 | | |
| miR-92a-3p | 25.03 | | |
| miR-93-5p | 28.20 | | |
| miR-181 | 27.40 | | |
| miR-206 | 29.02 | | |
| miR-324 | 30.57 | | |
| miR-486 | 20.15 | | |
| cel-miR-39-3p | 24.67 | | |
| RNU6-6P | 32.87 | | |
| SNORD95 | 30.57 | | |

Average Cycle Threshold (Ct) for miRNA measured in Serum from younger and older participants at baseline, post exercise and recovery. miRNA are separated into two columns, one showing miRNA that crossed the Ct for every participant at every time point (Expressed Consistently), and one showing miRNA that did not cross Ct for every participant at every time point (Not Expressed Consistently). [1]For points that miRNA did not cross Ct value was set at 40 (maximum number of cycles run) to quantify average Ct.

**Supplemental Table 3.** KEGG pathway analysis

| KEGG Pathway | *P* Value | Genes | miRNA |
|---|---|---|---|
| Proteoglycans in cancer | 3.05E-10 | 45 | 3 |
| Viral carcino is | 2.97E-05 | 36 | 3 |
| Glioma | 3.00E-05 | 19 | 3 |
| Melanoma | 7.53E-05 | 20 | 3 |
| Non-small cell lung cancer | 0.00013 | 18 | 3 |
| FoxO signaling pathway | 0.00026 | 34 | 3 |
| p53 signaling pathway | 0.00026 | 21 | 3 |
| Central carbon metabolism in cancer | 0.00027 | 18 | 3 |
| Prostate cancer | 0.00036 | 25 | 3 |
| Prolactin signaling pathway | 0.00068 | 19 | 3 |
| Estrogen signaling pathway | 0.00107 | 18 | 3 |
| Colorectal cancer | 0.00125 | 16 | 2 |
| Hepatitis B | 0.00125 | 33 | 3 |
| Thyroid hormone signaling pathway | 0.00173 | 30 | 3 |
| Transcriptional misregulation in cancer | 0.00173 | 32 | 3 |
| Bladder cancer | 0.00173 | 14 | 3 |
| Sphingolipid signaling pathway | 0.00540 | 26 | 3 |
| Cell cycle | 0.00540 | 30 | 3 |
| Progesterone-mediated oocyte maturation | 0.00756 | 22 | 3 |
| Chronic myeloid leukemia | 0.01095 | 17 | 3 |
| Pancreatic cancer | 0.01445 | 17 | 3 |
| Signaling pathways regulating pluripotency of stem cells | 0.01637 | 27 | 2 |
| HTLV-I infection | 0.01637 | 48 | 3 |
| Endometrial cancer | 0.01637 | 14 | 3 |
| PI3K-Akt signaling pathway | 0.02185 | 53 | 3 |
| Apoptosis | 0.02672 | 19 | 2 |
| Endocytosis | 0.03334 | 37 | 3 |
| ECM-receptor interaction | 0.03869 | 12 | 3 |
| AMPK signaling pathway | 0.04857 | 25 | 3 |

miR-19b-3p, miR-206, and miR-486 uploaded to DNA Intelligent Analysis (DIANA)-mirPath 3.0 (Alexander Fleming Biological Sciences Research Center (BSRC), Athens, Greece; http://diana.cslab.ece.ntua.gr).

**Supplemental Table 4.** Fold change in response to aging and acute exercise

| | | Baseline | Post Exercise | Recovery | Effect[1] | *P Value* |
|---|---|---|---|---|---|---|
| **miR-17b-5p** | YNG | 1.23 ± 0.18 | 1.32 ± 0.22 | 1.98 ± 0.40 | A | 0.03 |
| | OLD | 1.04 ± 0.18 | -1.19 ± 0.22 | -1.37 ± 0.40 | | |
| **miR-19a-3p** | YNG | 1.34 ± 0.21 | 1.68 ± 0.29 | 2.37 ± 0.50 | A | 0.01 |
| | OLD | 1.02 ± 0.21 | -1.18 ± 0.29 | -1.43 ± 0.50 | | |
| **miR-19b-3p** | YNG | 1.40 ± 0.18 | 1.74 ± 0.30 | 2.49 ± 0.56 | A | < 0.01 |
| | OLD | -1.08 ± 0.18 | -1.25± 0.30 | -1.50 ± 0.56 | | |
| **miR-20a-5p** | YNG | 1.25 ± 0.18 | 1.52 ± 0.26 | 2.19 ± 0.46 | A | 0.03 |
| | OLD | 1.02 ± 0.18 | -1.20 ± 0.26 | -1.27 ± 0.46 | | |
| **miR-24-3p** | YNG | 1.33 ± 0.23 | 1.63 ± 0.30 | 1.54 ± 0.30 | A | 0.04 |
| | OLD | 1.06 ± 0.23 | 1.26 ± 0.30 | -1.49 ± 0.30 | | |
| **miR-26a-5p** | YNG | 1.49 ± 0.30 | 1.82 ± 0.38 | 1.81 ± 0.44 | A | 0.04 |
| | OLD | 1.05 ± 0.30 | 1.18 ± 0.38 | -1.49 ± 0.44 | | |
| **miR-27a-3p** | YNG | 1.36 ± 0.24 | 1.82 ± 0.29 | 1.63 ± 0.27 | A | 0.02 |
| | OLD | 1.02 ± 0.24 | 1.01 ± 0.29 | 1.54 ± 0.27 | | |
| **miR-29a-3p** | YNG | 1.25 ± 0.19 | 1.57 ± 0.21 | 1.53 ± 0.26 | A | 0.03 |
| | OLD | 1.00 ± 0.19 | -1.10 ± 0.21 | -1.25 ± 0.26 | | |
| **miR-31-5p** | YNG | 1.01 ± 0.36 | -2.48 ± 0.20 | -1.25 ± 0.16 | T | 0.01 |
| | OLD | 1.77 ± 0.36 | -1.31 ± 0.20 | -1.41 ± 0.16 | | |
| **miR-93-5p** | YNG | 1.25 ± 0.19 | 1.36 ± 0.22 | 1.94 ± 0.42 | A | 0.04 |
| | OLD | 1.05 ± 0.19 | -1.10 ± 0.22 | -1.20 ± 0.42 | | |
| **miR-103a-3p** | YNG | 1.41 ± 0.28 | 1.59 ± 0.30 | 1.86 ± 0.40 | A | 0.03 |
| | OLD | 1.12 ± 0.28 | 1.04 ± 0.30 | -1.76 ± 0.40 | | |
| **miR-106b-5p** | YNG | 1.36 ± 0.19 | 1.52 ± 0.28 | 2.32 ± 0.53 | A | 0.02 |
| | OLD | -1.07 ± 0.19 | -1.31 ± 0.28 | -1.28 ± 0.53 | | |
| **miR-124-3p** | YNG | 1.00 ± 0.25 | -1.68 ± 0.18 | -1.12 ± 0.21 | T | 0.02 |
| | OLD | 1.62 ± 0.25 | 1.00 ± 0.18 | 1.64 ± 0.21 | | |
| **miR-130b-3p** | YNG | 1.30 ± 0.26 | 1.38 ± 0.23 | 1.59 ± 0.32 | A | 0.04 |
| | OLD | 1.10 ± 0.26 | 1.16 ± 0.23 | -1.48 ± 0.32 | | |
| **miR-143-3p** | YNG | 1.38 ± 0.26 | 1.79 ± 0.36 | 2.08 ± 0.36 | A | 0.01 |
| | OLD | 1.06 ± 0.26 | -1.22 ± 0.36 | -1.42 ± 0.36 | | |
| **miR-192-5p** | YNG | 1.33 ± 0.25 | 1.36 ± 0.29 | 1.90 ± 0.40 | A | 0.03 |
| | OLD | 1.08 ± 0.25 | -1.52 ± 0.29 | -1.77 ± 0.40 | | |
| **miR-195-5p** | YNG | 1.29 ± 0.20 | 1.44 ± 0.25 | 2.30 ± 0.46 | A | 0.02 |
| | OLD | 1.05 ± 0.20 | -1.23 ± 0.25 | 1.25 ± 0.46 | | |
| **miR-206** | YNG | 1.27 ± 0.60 | 1.05 ± 1.19 | 4.39 ± 1.23 | T | 0.05 |
| | OLD | 2.15 ± 0.60 | 3.11 ± 1.19 | 3.52 ± 1.23 | | |
| **miR-211-5p** | YNG | 1.11 ± 0.43 | -2.52 ± 0.19 | -1.18 ± 0.48 | T | 0.02 |
| | OLD | 1.96 ± 0.43 | -1.45 ± 0.19 | 1.65 ± 0.48 | | |
| **miR-221-3p** | YNG | 1.15 ± 0.30 | 1.36 ± 0.26 | 1.09 ± 0.24 | T | 0.03 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | OLD | 1.40 ± 0.30 | 1.64 ± 0.26 | 1.39 ± 0.24 | | |
| **miR-222-3p** | YNG | 1.23 ± 0.23 | 1.35 ± 0.26 | 1.23 ± 0.21 | T | 0.04 |
| | OLD | 1.15 ± 0.23 | 1.35 ± 0.26 | -1.52 ± 0.21 | | |
| **miR-324** | YNG | 1.29 ± 0.18 | 1.54 ± 0.31 | 2.04 ± 0.58 | A | 0.03 |
| | OLD | 1.04 ± 0.18 | 1.57 ± 0.31 | -1.68 ± 0.58 | | |
| **miR-374a-5p** | YNG | 1.77 ± 0.28 | 2.30 ± 0.47 | 2.42 ± 0.54 | A | < 0.01 |
| | OLD | 1.10 ± 0.28 | 1.08 ± 0.47 | -1.47 ± 0.54 | | |
| **miR-375** | YNG | 1.07 ± 0.37 | -1.62 ± 0.18 | -1.25 ± 0.24 | T | 0.03 |
| | OLD | 1.91 ± 0.37 | -1.21 ± 0.18 | 1.36 ± 0.24 | | |
| **miR-574-3p** | YNG | -1.05 ± 0.43 | -1.66 ± 0.26 | -1.72 ± 0.19 | A | 0.02 |
| | OLD | 1.97 ± 0.43 | 1.01 ± 0.26 | 1.36 ± 0.19 | | |

Mean ± SEM

Mixed model repeated measure ANOVA with Bonferonni Correction for pairwise comparisons

[1]Legend: A = Aging, T = Time, A x T = Aging-by-Time interaction

**Supplemental Table 5.** miRNA Component 1

| | Component Loading | Variance |
|---|---|---|
| **miR-19b-3p** | 1.019 | 60 % |
| **miR-195-5p** | 1.000 | |
| **miR-19a-3p** | 0.994 | |
| **miR-106-5p** | 0.978 | |
| **miR-20a-5p** | 0.959 | |
| **miR-17-5p** | 0.929 | |
| **miR-143-3p** | 0.924 | |
| **miR-26b-5p** | 0.876 | |
| **miR-18a-5p** | 0.875 | |
| **miR-93-5p** | 0.871 | |

Top 10 miRNA in Component 1 of Principal Component Analysis

**Supplemental Table 6.** Correlation of Component 1 miRNA to phosphorylation status of 6-hr post exercise Akt and S6K1

| | p-Akt$^{Ser473}$ | p-S6K1$^{Ser389}$ |
|---|---|---|
| **miR-17-3p** | $r = 0.794$ | $r = 0.466$ |
| | $P < 0.01$ | $P = 0.08$ |
| **miR-18a-5p** | $r = 0.500$ | $r = 0.339$ |
| | $P = 0.06$ | $P = 0.22$ |
| **miR-19a-3p** | $r = 0.814$ | $r = 0.600$ |
| | $P < 0.01$ | $P = 0.02$ |
| **miR-19b-3p** | $r = 0.796$ | $r = 0.514$ |
| | $P < 0.01$ | $P = 0.05$ |
| **miR-20a-5p** | $r = 0.832$ | $r = 0.539$ |
| | $P < 0.01$ | $P = 0.04$ |
| **miR-26b-5p** | $r = 0.702$ | $r = 0.524$ |
| | $P < 0.01$ | $P < 0.01$ |
| **miR-93-5p** | $r = 0.740$ | $r = 0.370$ |
| | $P < 0.01$ | $P = 0.175$ |
| **miR-106b-5p** | $r = 0.754$ | $r = 0.464$ |
| | $P < 0.01$ | $P = 0.08$ |
| **miR-143-3p** | $r = 0.786$ | $r = 0.567$ |
| | $P < 0.01$ | $P = 0.03$ |
| **miR-195-5p** | $r = 0.879$ | $r = 0.643$ |
| | $P < 0.01$ | $P = 0.01$ |

Associations determined using Spearman's Rho rank correlation coefficient

**Article 2: Circulating myomiR expression is upregulated after short-term energy restriction in older males**

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**Abstract**

Energy restriction (ER) attenuates protein synthesis. Recently, circulating muscle specific microRNA (c-myomiR) have been suggested to reflect rates of protein synthesis, however, their relationship remains undefined. The present investigation sought to determine the influence of energy restriction (ER) on c-myomiR expression and association to whole-body protein synthesis. Sixteen older (64 ± 2 yrs) overweight (28.5 ± 1.2 $kg \cdot m^{-2}$) males enrolled in this 35-day controlled feeding trial. A 7-day weight maintenance (WM) period was followed by 28 days of 30% ER; whole-body protein turnover ($^{15}$N-glycine) and c-myomiR (miR-1-3p, miR-133a-3p, miR-133b, miR-206) expression (RT-qPCR) was assessed at the conclusions of WM and ER. Participants lost ($P < 0.05$) 4.4 ± 0.3 kg body mass during ER. Overall, ER upregulated ($P < 0.05$) c-myomiR expression compared to WM. Expression of c-myomiR was inversely associated ($r = -0.700$, $P < 0.05$) with whole-body protein synthesis after ER. Similarly, *in vitro* analysis showed c-myomiR expression increased 2.9 ± 0.1 fold in the medium of C2C12 myotubes with a 2.1 ± 0.2 fold lower protein synthesis rate in energy-restricted compared to control cells. These novel *in vitro* and *in vivo* findings indicate that increased expression of c-myomiR reflect lower rates of protein synthesis following ER.

**Key Words**: Energy Deficit, whole-body protein turnover, microRNA

## Introduction

The need for adults 60 yrs and older to participate in weight loss interventions has become increasingly more evident, as 35% of this population is considered obese (1). Though weight loss improves metabolic and cardiovascular health, over time it can also result in undesirable reductions in skeletal muscle mass (2). The loss of muscle mass during energy restriction (ER; e.g., weight loss) is likely attributed, in part, to a diminished rate of whole-body and skeletal muscle protein synthesis (3-6). Reductions in protein synthetic rate during ER are due to endogenous protein stores being used in energy yielding processes and gluconeogenesis, rather than anabolism, resulting in a net negative protein balance (7, 8). For an older population who may already be experiencing age-associated losses in muscle mass (e.g., sarcopenia), lower rates of protein synthesis with ER may further compromise skeletal muscle mass and physical function (9).

Circulating microRNA (c-miRNA), small non-coding RNA present in blood, have been identified as potential noninvasive markers of physiological adaptions within skeletal muscle (10). Biogenesis of miRNA occurs in the nucleus of cells, where pre-miRNA are exported into the cytoplasm to become mature miRNA (11). From the cytoplasm mature miRNA can be released into circulation either actively in membrane-derived vesicles, or passively in apoptotic bodies (12). Though the exact mechanism by which c-miRNA influence cellular processes remains elusive, it has been observed that alterations in c-miRNA profiles reflect the underlying physiological condition of the tissue (13). In disease states that are known to blunt the rate of protein synthesis and diminish skeletal muscle mass, such as metabolic acidosis and Duchenne muscular dystrophy (14, 15),

upregulations in circulating skeletal muscle-specific miRNA (c-myomiR; miR-1-3p, miR-133a-3p, miR-133b, and miR-206) have been consistently reported as compared to healthy controls (16-20). Within skeletal muscle, myomiR are critical regulators of transcription factors, MyoD and myogenin, necessary for regeneration of myofibers (21), as well as the central pathway, mechanistic target of rapamycin complex 1 (mTORC1), controlling protein synthesis (22). While c-myomiR expression increases under pathophysiological conditions, whether expression of c-myomiR reflects rates of protein synthesis has not been examined.

The primary objective of this investigation was to determine the effect of short-term ER on c-myomiR expression and to examine their relationship to measurements of whole-body protein turnover. We hypothesized that ER would upregulate c-myomiR expression and be inversely associated with whole-body protein synthesis. While myomiR are highly expressed in skeletal muscle, comprising nearly 25% of all miRNA within the tissue (23), miR-1-3p, miR-133a-3p and miR-133b are present in other tissue. To assess if alterations in c-myomiR expression were reflective of changes in skeletal muscle, an *in vitro* experiment was conducted in C2C12 myocytes. We hypothesized that ER would reduce skeletal muscle protein synthesis, upregulate expression of c-myomiR in medium, and expression of myomiR within myotubes.

## Methods

### *Participants*

Participants were sedentary (< 2 exercise sessions per week), overweight (25-35 $kg \cdot m^{-2}$) males between 60 and 75 years of age. Participants were in good health and free of any chronic disease, as determined by medical screening. For enrollment into this

investigation participants were willing to consume only foods and beverages provided by Human Nutrition Research Center on Aging (HNRCA) metabolic kitchen, abstain from alcohol, tobacco, and dietary supplement use, and maintain their level of physical activity. The Tufts University Health Sciences Campus Institutional Review Board approved this investigation, with informed written consent obtained from all participants.

***Study Design***

For this 35-day controlled feeding intervention, subjects were placed on a eucaloric diet (weight maintenance; WM) for the first 7 days. Nitrogen balance (NBAL), whole-body protein turnover (WBPTO) and resting metabolic rate (RMR) were measured at the end of WM. Starting on Study Day 8 energy intake was reduced by 30% of total energy needs for the remaining 28 days. Dietary protein intake was set at 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ and dietary fat and carbohydrate were manipulated to reduce energy intake. Measures of WBPTO, NBAL and RMR were repeated at the end of the ER phase (NBAL was also measured on day 13 to determine the initial adaptions to energy restriction). An additional arm of this investigation was that participants received 90 $mmol \cdot d^{-1}$ $KHCO_3$ or a matched placebo for the 28 days of ER. No effect of $KHCO_3$ was observed on outcome variables so data were collapsed for analysis of the effect of energy status on independent variables. Participants reported to the Human Nutrition Research Center on Aging HNRCA 2-3 times per week to pickup study food and beverage and weighed to assess compliance.

***Anthropometrics***

Height was measured in duplicate to the nearest 0.1 cm using a stadiometer. Body mass was measured using a calibrated digital scale (Seca, Los Angeles, CA, USA) to the nearest 0.1 kg at baseline to confirm study eligibility and daily during WM and ER.

***Diet***

Energy needs were individualized for each participant using the Harris Benedict equation with a fixed factor of 1.2 to account for dietary thermogenesis and daily living activities (e.g., walking, brushing teeth, etc). The 7-day WM phase allowed time for adaptation to the diet prescription. Dietary protein was set at 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ to be consistent with recommendations from the PROT-AGE Study Group, that states older individuals' protein requirements are above the current RDA of 0.8 $g \cdot kg^{-1} \cdot d^{-1}$ and should consume at least 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ (24). Dietary fat accounted for 25-30% of total energy and carbohydrate provided the remainder of the prescribed energy. Energy intake was reduced by 30% of total energy needs at the end of the WM phase. Dietary protein intake remained constant at 1.0 $g \cdot kg^{-1} \cdot d^{-1}$, fat accounted for 25-30% of total energy and carbohydrate provided the remainder of the prescribed energy. Meals were provided in 3-d menu cycles and prepared in the HNRCA metabolic kitchen. Each meal was prepared in advance and checked for accuracy by study staff. Participants were provided with a multivitamin/mineral supplement to ensure that micronutrient requirements were met. Energy and macronutrient intakes were recorded and analyzed using Nutrition Data System for Research (University of Minnesota; **Table 1**)

***Nitrogen Balance***

Total nitrogen content of the urine was determined on days 6, 13 and 34 from a single pooled 24-hr urine sample using pyrochemiluminescence. Urinary creatinine was

measured using the Jaffe reaction to verify complete urine collections. Apparent nitrogen balance was calculated as the difference of nitrogen intake minus urinary nitrogen excretion plus miscellaneous (estimated at 5 $mg \cdot kg^{-1}$) and fecal (estimated at 2 $g \cdot d^{-1}$) losses (25). Urinary creatinine was measured on an automated clinical chemistry analyzer (Olympus AU400, Olympus America Inc., Melville, NY). Total nitrogen excretion was measured using a model FP-2000 nitrogen/protein determinator (LECO, St. Joseph, MI).

***Resting Metabolic Rate***

Resting metabolic rate (RMR) was measured using open circuit indirect calorimetry (Parvo Medics TrueOne 2400, Sandy, UT) at the end of WM (Study Day 7) and ER (Study Day 35). Measurements were conducted between 0600-0800-hrs after a 10-hr overnight fast. Participants rested in the supine position for approximately 30-min before each measurement in a quiet, dim, temperature regulated room. An acrylic hood was placed over the participant's head to collect all expired air during the test. Participants were instructed to remain still, stay awake, and breath normally during the collection period. The test was discontinued when 20-min of steady-state oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) were recorded.

***Substrate Oxidation***

Nitrogen values determined by the 24-hr urine collections coincided with RMR measurements to estimate substrate oxidation according to Ferrannini (26).

Protein oxidation ($g \cdot d^{-1}$) = 6.25 x urinary nitrogen (g)

Glucose oxidation ($g \cdot d^{-1}$) = 4.12 x $VCO_2$ – 2.91 x $VO_2$ – 2.56 x urinary nitrogen (g)

Fat oxidation ($g \cdot d^{-1}$) = 1.69 x $VCO_2$ – 1.69 x $VO_2$ – 1.92 x urinary nitrogen (g)

***Blood Sampling***

Blood samples were obtained at the end of the WM and ER periods after an overnight fast. Blood samples were allowed to clot at room temperature and then centrifuged at 2,135g for 10 minutes at 4°C. Serum was derived and stored at −80°C until analyzed.

***Substrate and Hormone Analysis***

Serum glucose and triglyceride concentrations were assessed using a Beckman Coulter AU400e Chemistry analyzer (Beckman Coulter, Inc., Brea CA). Insulin concentrations were determined using the 2470 Wizard$^2$ Automatic Gamma Counter (PerkinElmer, Cambridge MA) and Human-Specific RIA Insulin kits (Millipore, Bilerica, MA).

***Whole-Body Protein Turnover***

Whole-body protein turnover was assessed for a 24-hr period starting on Study Days 6 and 34 using the 'End-Product' method (27). Stable isotope, $^{15}$N-glycine, was administered in the morning following a 12-hr overnight fast. Immediately before each isotope dosing subjects provided a baseline urine sample for determination of background $^{15}$N-nitrogen and completely emptied their bladders. A single oral dose of $^{15}$N-glycine (300 g; Cambridge Isotope Laboratories, Andover, MA) was dissolved in bottled water and then administered, (28). Urine was then collected for 24-hrs after dosing. The $^{15}$N enrichment of urinary nitrogen (ratio of tracer to tracee, t:t) was determined using isotope ratio mass spectroscopy (Metabolic Solutions, Nashua, NH). The t:t ratio for the cumulative sample was corrected for the background $^{15}$N-nitrogen enrichments. Nitrogen intake (I) was determined from analysis of 24-hr food consumption. Nitrogen flux (Q), protein synthesis (PS), protein breakdown (PB), and net protein balance (NET) was

calculated using the following equations, where D denotes the oral dose of $^{15}N$ (D = g glycine x 0.1972).

Q (g $N \cdot kg^{-1} \cdot d^{-1}$) = [D/(corrected t:t)/24 hr • body weight]

PS ($g \cdot kg^{-1} \cdot d^{-1}$) = [Q – (E/24 hr • body weight)] • 6.25

PB ($g \cdot kg^{-1} \cdot d^{-1}$) = [Q – (I/24 hr • body weight)] • 6.25

NET ($g \cdot kg^{-1} \cdot d^{-1}$) = PS – PB

***Circulating microRNA Extraction and Expression***

Circulating miRNA were extracted from 200 µL serum using an miRNeasy Serum/Plasma kit, which allows for extraction and purification of small (< 200 nt) cell-free RNA (217184; Qiagen Valencia, CA). Before extracting RNA, samples were centrifuged for 10 min at 4°C to remove potential contaminating cellular debris. Due to the small amount of RNA in the serum, 3.5 µL of a Spike-In Control (C. elegans miR-39; 219610; QIAGEN) was added to all samples before RNA extraction to determine the yield of template recovered.

c-miRNA of interest (miR-1, miR-133a-3p, miR133b, miR-206) were analyzed using TaqMan® MicroRNA Assays (4427975;Applied Biosystems) using a multiplex RT and pre-amplification protocol (29). miRNA were reverse-transcribed using the TaqMan® microRNA RT kit (4366596; Applied Biosystems) with the miRNA-specific stem-loop RT primers pooled in 1X-Tris-EDTA (TE) buffer for a final dilution of 0.05X for each miRNA RT primer. The RT primer pool (6 µl) was added to the RT reaction mix (0.3 µl 100mM dNTP, 3 µl enzyme, 1.5 µl 10× RT buffer, 0.19 µl RNase inhibitor) and 3 µl of serum RNA. A pre-amplification step was performed to increase cDNA template using a primer pool of 20 X Taqman® Small RNA Assay for the miRNA of interest at 0.05X

concentration in 0.1X TE buffer. Pre-amplification reaction mix was constituted of 3.75 µl primer pool, 2.5 µl cDNA, 12.5 µl Taqman® Universal PCR Master Mix (2X), no UNG (#4440040 Applied Biosystems) and 6.25 µl nuclease free $H_2O$. Reverse transcription and pre-amplification were conducted using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). RT-qPCR amplifications were conducted using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

All miRNA were normalized to the geometric mean of Spike-In Control miR-39 (external control) and U6 (internal control). Normalizing miRNA to the geometric mean of external and internal controls allows correction of both technical and inter-individual variations. Three potential internal controls (U6, U44, and U48) were assessed for this analysis. U6 was determined to be the most stable and least variable internal control in the present investigation. Geometric mean of Spike-In Control miR-39 and U6 was determined to be a homogenously and stably expressed housekeeper with a coefficients of variation (CV) of 4% (30). Fold changes were calculated using the ΔΔ cycle threshold ($\Delta\Delta C_T$) method (31).

***Cell Culture***

To investigate if altered expression of circulating myomiRs was the result of adaptations in skeletal muscle, we designed a cell culture experiment to assess the pattern of miRNA expression in circulation (media) and cellular level following acute energy restriction. Stock C2C12 (mouse) myoblasts (American Type Culture Collection, Manassas, VA, USA) were maintained at 37°C (95% O2-5% CO2) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) culture medium, 1% penicillin–streptomycin (Sigma-Aldrich), and 5.5

mM glucose. Once myoblasts were 80% confluent, they were seeded in two 6-well plates, one plate of Western blotting and one plate for PCR. Myoblasts were differentiated to myotubes for 4 days in medium containing 2% horse serum (Sigma-Aldrich). Following differentiation, 3-wells were maintained in standard culture medium containing 2% horse serum to serve as controls, and 3-wells were maintained on a glucose free culture medium (RPMI-1640 Medium; Sigma-Aldrich) containing 2% horse serum to mimic CR for 48 hrs. The 48-hr incubation period in glucose free medium has previously been reported to adequate to downregulate mTORC1 signaling (32).

Prior to harvesting, C2C12 cells used for Western blotting were serum starved for 2-hrs. After serum starve, 1 µM of puromycin was added to each well for 30 min to allow for estimation of protein synthetic rate (33). Phosphorylation status and total protein expression of molecular markers associated with skeletal muscle protein synthesis and myogenesis were determined using Western blotting. Homogenates used for muscle protein content estimations were centrifuged for 15 min at 10000 x g at 4°C, the supernatant (lysate) was collected and protein concentration analyzed (ThermoFisher). Muscle lysates were solubilized in Laemmli buffer and separated by SDS-PAGE using precast Tris·HCl gels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to polyvinylidene fluoride membranes and exposed to commercially available primary antibodies specific to puromycin (Millipore; Billerica, MA, USA), mTOR, p-mTOR$^{Ser2448}$, rpS6, p-rpS6$^{Ser235/236}$ (Cell Signaling Technology, Danvers, MA, USA), and MyoD and myogenin (Santa Cruz, Dallas, TX USA) at 4 °C overnight. Labeling was performed using secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology), and chemiluminescent reagent was applied

(Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Blots were quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad). Phosphorylation status was normalized to total protein to determine the ratio of phosphorylation-to-total protein. Heat shock protein 90 (HSP90) was used to confirm equal protein loading to normalize puromycin, MyoD, and myogenin. All data are presented as fold change of energy restricted cells relative to the mean values for control wells (normal medium).

To assess alterations of c-myomiR, 200 µL medium was collected following the 48-hr incubation period. Extraction and expression of c-myomiR were performed as described above. Total RNA was isolated from C2C12 cells using mirVana$^{TM}$ miRNA isolation kit (Invitrogen, Carlsbad, CA, USA) following instructions for total RNA extraction. RNA quantity and quality were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA (250 ng) were synthesized into cDNA (iScript$^{TM}$ Advanced cDNA Synthesis Kit; Bio-Rad). Samples were run in 20 µl reactions in triplicates, using Taqman® Univsersal PCR MasterMix (2X), no UNG (Applied Biosystems). All miRNA were normalized to B2M. Fold changes for miRNA were calculated using the ΔΔ cycle threshold ($\Delta\Delta C_T$) method (31) with fold change of energy restricted cells expressed relative to the mean values for the control wells (normal medium).

***myomiR Score***

To group the four myomiRs (miR-1, miR-133a-3p, miR133b, miR-206) assessed *in vivo* in circulation and *in vitro* in myotubes, a myomiR Score was calculated as the median fold change for all myomiR (34).

### *Statistical Analysis*

Normality was determined using Shapiro Wilk tests for dependent variables. Expression of c-myomiR were not normally distributed in the human trial. Fold change data for c-myomiR were log transformed ($log_2$) for statistical analysis. Student's paired t-test was used to assess differences between WM versus ER values for RMR, substrate oxidation, metabolic profile, WBPTO and c-miR expression. Repeated measures ANOVA were used to assess NBAL. Backward linear regression analysis of whole-body protein synthesis, breakdown, and carbohydrate, fat and protein oxidation was conducted to determine the strongest predictor of c-myomiR score. Spearman's rank correlation coefficient was used to determine the correlation of c-myomiR scores to measurements of whole-body protein synthesis. Student's unpaired t-test were used to assess differences between control and energy restricted cells for Western blots and myomiR expression. Significance was set at $P < 0.05$. Data were analyzed using IBM SPSS Statistics for Windows (version 22.0; IBM Corp., Armonk, NY).

## Results

### *Participant Characteristics and Weight Loss*

Sixteen males (64 ± 2 years) participated in the study. Baseline body mass, height, and body mass index (BMI) were 89.3 ± 2.2 kg, 1.8 ± 0.4 m, and 28.5 ± 1.2 $kg \cdot m^{-2}$, respectively. Body mass was stable during the 7-d WM period with a change of < 1% (88.8 kg) from Study Day 1 to 7. The mean energy deficit during the 28-day ER period was 31 ± 1%, resulting in declines ($P < 0.05$) of body mass (4.4 ± 0.8 kg) and BMI (1.4 ± 0.3 $kg \cdot m^{-2}$; **Figure 1**).

### *Metabolic Adaptions*

At the conclusion of ER, RMR declined ($P < 0.05$) 73 ± 31 kcal compared to WM (**Table 2**). Similarly, respiratory quotation was reduced ($P < 0.05$) 0.05 ± 0.01 ($VCO_2 \cdot VO_2^{-1}$) resulting in a 18 ± 2% decrease ($P < 0.05$) in whole-body carbohydrate oxidation and an 18 ± 3% increase ($P < 0.05$) in whole-body fat oxidation during ER compared to WM. Energy restriction did not alter whole-body protein oxidation. Insulin concentrations were 15.0 ± 6.1 $pmol \cdot L^{-1}$ lower ($P < 0.05$) during ER compared to WM, which resulted in a decline of HOMA-IR of 0.6 ± 0.2 (**Table 2**). Triglyceride concentrations were 30.9 ± 10.7 $mg \cdot dL^{-1}$ lower ($P < 0.05$) during ER compared to WM values. No effect of energy status was observed in the circulating glucose concentrations.

***Nitrogen Balance Whole-Body Protein Turnover***

Compared to WM, NBAL was most negative ($P < 0.05$; -1.07 ± 0.74 $g \cdot d^{-1}$) during the initial phase of ER (Study Day 13), but returned to values similar to WM (-0.04 ± 0.62 $g \cdot d^{-1}$) at the end of ER. Similarly, compared to WM change in whole-body protein flux (0.04 ± 0.02 g N $g \cdot kg^{-1} \cdot d^{-1}$), synthesis (0.26 ± 0.15 g protein $g \cdot kg^{-1} \cdot d^{-1}$), breakdown (0.22 ± 0.16 g protein $g \cdot kg^{-1} \cdot d^{-1}$) and net (0.04 ± 0.2 g protein $g \cdot kg^{-1} \cdot d^{-1}$) during ER was not different.

***Circulating myomiR Expression***

Energy restriction resulted in an upregulation ($P < 0.05$) in c-myomiR score, with significant increases in the expression of miR-133a and miR-133b compared to WM (**Figure 2A**). Expression of miR-1-3p tended ($P = 0.07$) to be higher during ER compared to WM. No effect of energy status was observed for the expression of miR-206. Backward linear regression analysis of whole-body protein synthesis, breakdown, and carbohydrate, fat and protein oxidation determined protein synthesis to be the

strongest predictor of c-myomiR, explaining 48% of the variance. An inverse association ($P < 0.05$) was observed with ER c-myomiR scores and whole-body protein synthesis ($r = -0.700$, $r^2 = -0.512$; **Figure 2B**). Additionally, delta (ER – WM) whole-body protein synthesis was inversely associated ($r = -0.544$, $r^2 = -0.29$, $P < 0.05$) with c-myomiR score (**Figure 2C**).

***Cell Culture***

Protein synthetic rate was downregulated ($P < 0.05$) 2.1 ± 0.2 fold in energy-restricted cells compared to control (**Figure 3A**). Phosphorylation status (i.e., activity) of p-mTORC1$^{Ser2448}$ and p-rpS6$^{Ser235/236}$ were 2.1 ± 0.3 and 13.9 ± 1.5 fold lower ($P < 0.05$), respectively, in energy-restricted cells versus control cells. Energy restriction diminished ($P < 0.05$) total protein expression of myomiR transcription factors, MyoD and myogenin, 1.4 ± 0.1 and 2.2 ± 0.2 fold, respectively, compared to control.

Expression of c-myomiR in the medium of isolated C2C12 myotubes were upregulated ($P < 0.05$) in response to energy restriction, with the overall c-myomiR score being 2.9 ± 0.1 fold higher compared to control (**Figure 3B**). Furthermore, with upregulation of c-myomiRs there was a downregulation ($P < 0.05$) of myomiR expression within cells in response to energy restriction (**Figure 3C**).

**Discussion**

Primary findings from the present investigation were that short-term ER (28-day; 30% total energy requirements) increased the expression myomiR in circulation, which were inversely associated with 24-hr measures of whole-body protein synthesis. Confirmation of an upregulation in c-myomiR expression in the media of isolated myotubes with lower rates of skeletal muscle protein synthesis following ER *in vitro* strongly supports the

hypothesis that alterations in c-myomiR reflect physiological adaptions within skeletal muscle. Together results from both *in vivo* and *in vitro* analysis provide compelling evidence that increased expression of c-myomiR reflect lower rates of protein synthesis following ER.

For older individuals engaging in weight loss interventions, understanding the physiological consequences of ER is critical to identify appropriate strategies to minimize muscle loss. Determination of noninvasive markers that reflect skeletal muscle adaption could potentially allow for understanding of physiological adaptions to nutrient and exercise interventions. Within skeletal muscle, miRNA have been demonstrated to be important regulators of muscle mass and phenotype (35, 36), interacting with molecular pathways that influence myofiber regeneration, hypertrophy and metabolism (37). Discovery of skeletal muscle specific miRNA in circulation (c-myomiR) has created much interest into their potential functional role and use as a noninvasive biomarker of the physiological state of muscle tissue. Recently, our laboratory (unpublished data) observed that aging upregulates miR-1-3p, miR-133a-3p, and miR-206 expression by ≥ 1.5 fold in older compared to younger adults. Furthermore, an inverse correlation was observed with miR-133a-3p ($r$ = -0.61) and miR-206 ($r$ = -0.51) expression compared to percent fat-free mass (unpublished data). The main outcome from the present investigation suggests that ER may further exacerbate dysregulation of c-myomiR with aging. Supporting results from the human trial, ER induced reduction of protein synthesis *in vitro* resulted in a similar upregulation of c-myomiR within the media of isolated myotubes. These confirmatory findings establish that c-myomiR are pertinent

noninvasive markers of physiological adaptions to nutrition interventions within skeletal muscle.

Altered expression c-myomiR have previously been observed to reveal the pathophysiological state of skeletal muscle (17-20), however, no relationship to alterations in protein synthesis has been investigated. Findings from the present investigation show that upregulation of c-myomiR reflect lower rates of protein synthesis after ER in both *in vivo* and *in vitro* models. While an inverse correlation was observed between c-myomiR and whole-body protein synthesis, no effect of energy was observed on whole-body protein synthesis. Previous investigations have reported short-term ER results in diminished rates of protein synthesis (3, 38-40). Discordant results in the present investigation can likely be explained by methodological discrepancies. Specifically, past studies were primarily conducted in the fasted state under resting conditions, while the current investigation assessed whole-body protein synthesis over a 24-hr period. The inclusion of dietary intake, particularly protein, and activities of daily living are known to influence the rate of protein synthesis during ER (41, 42). It is not surprising that the addition of these factors would result in differing findings from past investigations conducted under fasted, resting conditions. Despite the lack of an energy effect on whole-body protein synthesis, findings from this investigation show elevations in c-myomiR expression reflect lower rate of whole-body protein synthesis. Furthermore, elevations in c-myomiR with ER induced reduction in protein synthesis were confirmed in our *in vitro* analysis.

*In vitro*, upregulation of c-myomiR with ER also resulted in diminished expression of myomiR within myotubes. Contrary to findings from the present investigation, during

periods of increased protein synthesis resulting in skeletal muscle hypertrophy, myomiR expression has previously been shown to be downregulated (43). Specifically, miR-1 and miR-133a inhibit the expression of upstream targets of mTORC1, IGF-1 and IGF-1R (44). During periods of muscle growth induced by mechanical load, it is believed that down regulation of miR-1 and miR-133a allows for increased activation of mTORC1 through IGF-1, resulting in increased rates of protein synthesis (22). It would thus be anticipated that with decreased rates of protein synthesis miR-1 and miR-133a expression would be increased. The conflicting results observed in the present *in vitro* analysis could likely be attributed to the fact that a single miRNA can target hundreds of different genes (10). While mechanical load may diminish myomiR expression allowing for increased mTORC1 signaling, there is potential that ER induced alterations in myomiR may target other markers within the mTORC1 pathway or an alternative pathway associated with protein synthetic rate. Supporting this, ER in the present study resulted in lower translation of myogenic transcription factors, MyoD and myogenin, which are fundamental to the synthesis and regeneration of myofibers. This findings is corroborated by past work showing that miR-1 and miR-206 regulate myofiber regeneration, with inhibition of miR-1 and miR-206 downregulating MyoD and myogenin expression to blunt myogenesis (21).

Though results from the present investigations provide new insight into the relationship of c-myomiR expression and protein synthesis, they are not without limitations. The lack of assessment of changes in body composition is a clear limitation. Not having this applied outcome limits our ability to determine if increased expression of c-myomiR not only reflects lower rates of protein synthesis, but reductions in fat-free

mass. Additionally, while our investigation established an association between c-myomiR expression and whole-body protein synthesis, it is important to note that skeletal muscle only contributes 30-45% whole-body protein turnover (45). Assessment of skeletal muscle protein synthesis would be required to determine if c-myomiR reflects alterations in protein synthesis at the skeletal muscle level. While a more comprehensive analysis may have been conducted had additional measurements been included, utilization of both *in vivo* and *in vitro* analysis this investigation clearly establish a relationship between elevations in c-myomiR being with lower rates of protein synthesis.

In conclusion, this study links an increased expression of c-myomiR to lower rates of protein synthesis after 28 days of 30% ER in older males. Corroboration of findings from the human trial by myotubes in our cell culture experiment provide further evidence that ER induced alterations in c-myomiR are reflective of skeletal muscle. While these results establish a relationship between c-myomiR and protein synthesis, further investigation is warranted to determine the functional role that modification in c-myomiR have on protein synthetic rate at the whole-body and skeletal muscle level. Though causation cannot be determined, findings from *in vivo* and *in vitro* analysis suggest that upregulation in expression of myomiR may be noninvasive markers of ER induced reductions in the rate of protein synthesis.

**Conflict of Interest**

The authors report no conflicts of interest.

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**Table 1.** Dietary intake

| | Weight Maintenance | Energy Restriction | Delta |
|---|---|---|---|
| **Absolute Intake** | | | |
| Energy Intake ($kcal \cdot d^{-1}$) | 2611 ± 153 | 1810 ± 51 | -800 ± 34 |
| Carbohydrate ($g \cdot d^{-1}$) | 404 ± 11 | 257 ± 9 | -147 ± 7 |
| Fat ($g \cdot d^{-1}$) | 77 ± 2 | 51 ± 1 | -27 ± 1 |
| Protein ($g \cdot d^{-1}$) | 89 ± 2 | 89 ± 2 | -0.2 ± 0.1 |
| **Relative Intake** | | | |
| Energy Intake ($kcal \cdot kg^{-1} \cdot d^{-1}$) | 29.46 ± 0.52 | 21.5 ± 0.5 | -7.97 ± 0.27 |
| Carbohydrate ($g \cdot kg^{-1} \cdot d^{-1}$) | 4.56 ± 0.09 | 3.05 ± 0.09 | -1.51 ± 0.06 |
| Fat ($g \cdot kg^{-1} \cdot d^{-1}$) | 0.87 ± 0.02 | 0.60 ± 0.01 | -0.27 ± 0.01 |
| Protein ($g \cdot kg^{-1} \cdot d^{-1}$) | 1.00 ± 0.01 | 1.05 ± 0.01 | 0.05 ± 0.01 |

Mean ± SEM

**Table 2.** Metabolic adaptions

| | **Weight Maintenance** | **Energy Restriction** | ***P* Value** |
|---|---|---|---|
| RMR (kcal) | 1586 ± 23 | 1513 ± 22 | < 0.01 |
| RQ ($VCO_2 \cdot VO_2^{-1}$) | 0.85 ± 0.02 | 0.80 ± 0.02 | < 0.01 |
| **Substrate Utilization (%)** | | | |
| Carbohydrate | 45 ± 2 | 27 ± 3 | < 0.01 |
| Fat | 40 ± 2 | 58 ± 2 | < 0.01 |
| Protein | 15 ± 1 | 15 ± 1 | 0.88 |
| **Metabolic Profile** | | | |
| Glucose ($mmol \cdot L^{-1}$) | 5.2 ± 0.1 | 5.2 ± 0.1 | 0.78 |
| Insulin ($pmol \cdot L^{-1}$) | 77.3 ± 7.8 | 62.2 ± 7.7 | 0.03 |
| HOMA IR | 2.6 ± 0.3 | 2.1 ± 0.3 | 0.04 |
| Triglyceride ($mg \cdot dL^{-1}$) | 132.6 ± 13.4 | 101.7 ± 10.5 | < 0.01 |

Mean ± SEM, RMR: resting metabolic rate, RQ: respiratory quotient

**Figure Legends**

**Figure 1:** Change in body mass and body mass index from weight maintenance (Study Day 7). *Different from Day 7; $P < 0.05$.

**Figure 2:** Expression of c-myomiR during weight maintenance (□) and energy restriction (■) (**A**). *Different from weight maintenance; $P < 0.05$. Correlation of c-myomiR Score to energy restriction whole-body protein synthesis (**B**) and delta (energy restriction – weight maintenance) whole-body protein synthesis (**C**). *Different from weight maintenance; $P < 0.05$.

**Figure 3**: Difference in molecular regulators of muscle hypertrophy and myogenesis in control (□) and energy restricted (■) myotubes (**A**). Puromycin, MyoD and myogenin normalized to HSP90. p-mTOR$^{Ser2448}$ and p-rpS6$^{ser235/236}$ normalized to total mTOR and rpS6, respectively. Expression of c-myomiR in the medium of wells (**B**). Expression of myomiR within myotubes (**C**). *Different from control; $P < 0.05$.

**Figure 1.** Change in body mass and body mass index

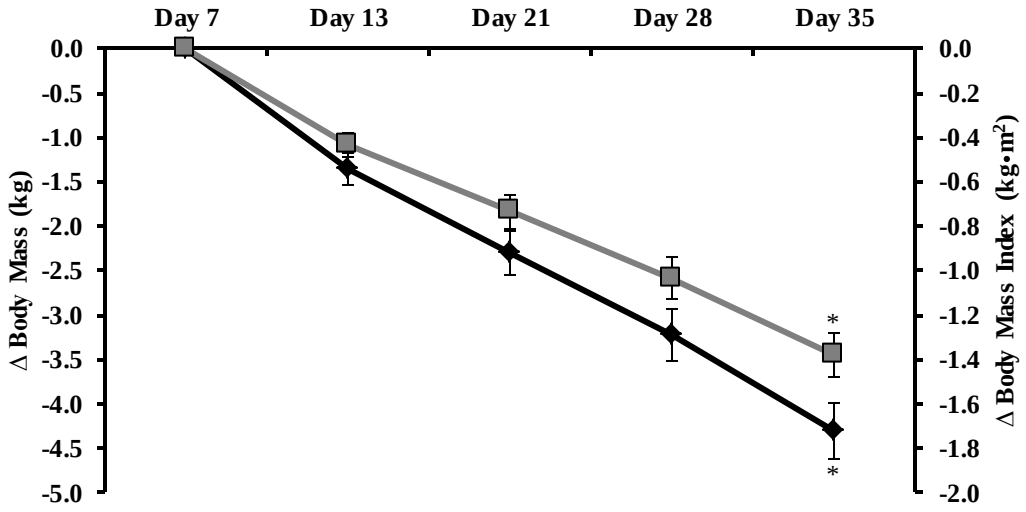


**Figure 2.** c-myomiR expression and association to protein synthesis

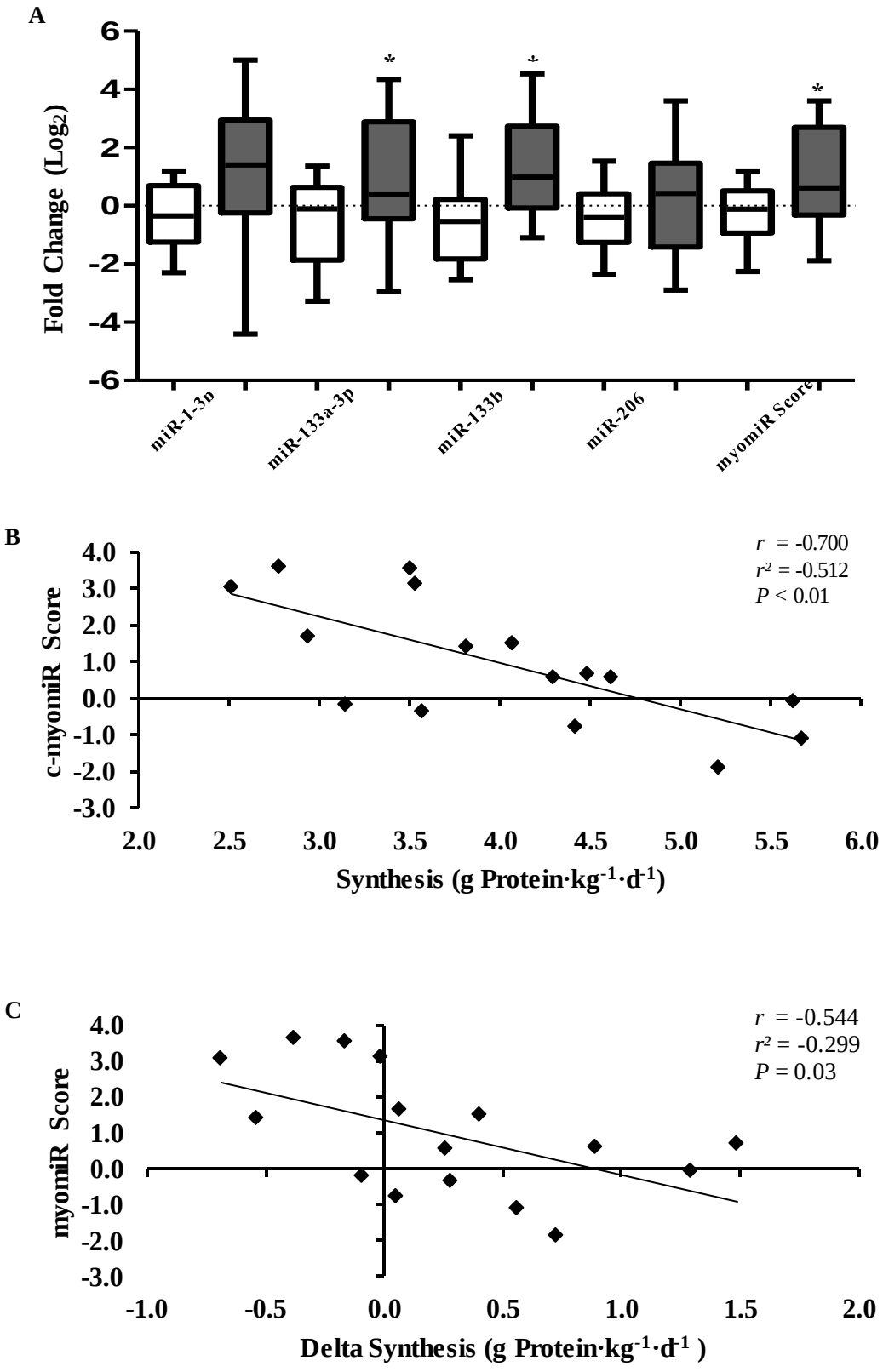
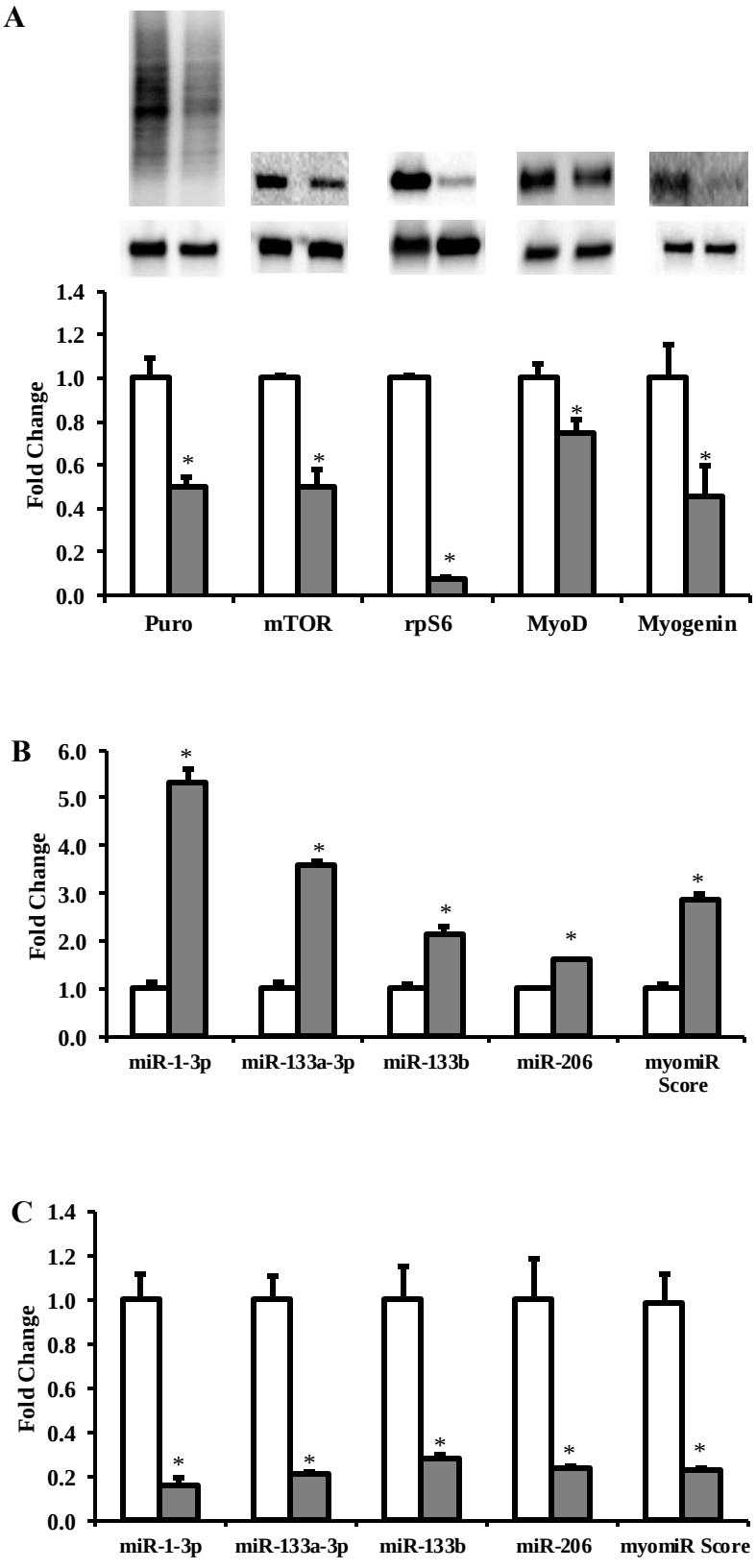


**Figure 3.** *In vitro* c-myomiR analysis



**Article 3: Prolonged calorie restriction downregulates skeletal muscle mTORC1 signaling independent of dietary protein intake and associated microRNA expression**

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**Abstract**

Short-term (5–10 days) calorie restriction (CR) downregulates muscle protein synthesis, with consumption of a high protein-based diet attenuating this decline. Benefit of increase protein intake is believed to be due to maintenance of amino acid-mediated anabolic signaling through the mechanistic target of rapamycin complex 1 (mTORC1), however, there is limited evidence to support this contention. The objective of this investigation was to determine the effects of prolonged CR and high protein diets on skeletal muscle mTORC1 signaling and the expression of associated microRNA (miR). 12-wk old male Sprague Dawley rats consumed ad libitum (AL) or calorie restricted (CR; 40%) adequate (10%, AIN-93M) or high (32%) protein milk-based diets for 16 weeks. Body composition was determined using dual energy X-ray absorptiometry and muscle homogenate protein concentrations expressed relative to fat-free mass to estimate muscle protein content. Western blot and RT-qPCR were used to determine mTORC1 signaling and mRNA and miR expression in fasted mixed gastrocnemius. Independent of dietary protein intake, muscle protein content was 38% lower ($P < 0.05$) in CR compared to AL. Phosphorylation and total Akt, mTOR, rpS6 and p70S6K were lower ($P < 0.05$) in CR versus AL, and total rpS6 was associated with muscle protein content ($r = 0.64$, $r^2 = 0.36$). Skeletal muscle miR expression was not altered by either energy or protein intake. This study provides evidence that chronic CR diminishes muscle protein content by downregulating mTORC1 signaling. This response is independent of skeletal muscle miR and dietary protein.

**Key Words:** Muscle protein content, energy deficit, rpS6, miR-99, miR-100

## Introduction

Calorie restriction (CR) is a strategy to lose body fat and reduce total body mass. However, skeletal muscle is also lost during CR, which may compromise successful weight management and negatively impact physical function. The loss of skeletal muscle may be due in part to the effects of CR on muscle protein synthesis (MPS) (Pasiakos et al., 2015). Recent studies have demonstrated that CR, particularly short-term CR (≤ 21 days), downregulates fasting MPS and blunts anabolic sensitivity to a protein-containing meal (Pasiakos et al., 2010;Areta et al., 2014;Hector et al., 2015;Murphy et al., 2015). Increasing dietary protein intake above the recommended dietary allowance (RDA; 0.8 $g \cdot kg^{-1} \cdot d^{-1}$) may attenuate declines in MPS, preserve anabolic sensitivity to a protein-containing meal, and spare skeletal muscle mass during short-term CR (Pasiakos et al., 2013;Areta et al., 2014). The anabolic sparing effects of high protein diets during short-term CR are likely mediated by the mechanistic target of rapamycin complex 1 (mTORC1) (Proud, 2007;Drummond et al., 2009;Pasiakos, 2012), although evidence to support this contention is limited (Pasiakos et al., 2010;Miller et al., 2013;Areta et al., 2014). Despite changes in MPS, altered mTOC1 signaling under fasting conditions has not been shown during short-term CR with adequate or high protein diets. Whether prolonged CR and dietary protein level affect mTORC1 signaling, and result in modifications in fat-free mass has not been determined.

Recently, microRNA (miR) have been identified as novel regulators of skeletal muscle mass (Baek et al., 2008;Selbach et al., 2008;Rivas et al., 2014), with specific miR serving as negative regulators of hypertrophy through mRNA degradation or repression of translation for mTORC1 associated signaling proteins (Pillai et al., 2004;Kong et al.,

2008). In acute cell culture models, overexpressing members of the miR-99/100 family inhibits gene expression and protein translation of mTORC1 signaling proteins, downregulating the activity of the pathway and ultimately cellular proliferation (Jin et al., 2013;Wei et al., 2013;Jia et al., 2014). In human muscle, diminished miR-99 and miR-100 expression predict the anabolic response to a resistance exercise bout, with reduced expression of these miR upregulating mTORC1 signaling (Zacharewicz et al., 2014). While acute alterations in miR expression have been associated with skeletal muscle mTORC1 signaling in some studies, no investigations have assessed whether prolonged CR and dietary protein level augment miR expression, subsequent translational modifications in mTORC1 signaling, and whether miR are associated with skeletal muscle mass in response to underfeeding.

The objectives of this study were to: 1) determine the effects of prolonged CR on mTORC1 signaling, 2) determine the extent to which high protein diets modulate the mTORC1 response, 3) assess whether CR and dietary protein-mediated changes in mTORC1 signaling influence skeletal muscle, and 4) explore the potential regulatory role of miR. We hypothesized that prolonged CR would downregulate mTORC1 signaling resulting in lower fat-free mass after CR. We expected that consuming a high protein diet would prevent declines in mTORC1 signaling and spare fat-free mass. We hypothesized that CR and dietary protein-mediated changes in mTORC1 signaling would result from translational modifications secondary to altered expression of associated miR.

**Experimental design**

Twelve-week-old male Sprague Dawley rats ($n$ = 40; Charles River Laboratories) were randomized by body mass to one of four diet groups consuming *ad libitum* (AL) or

calorie restricted (CR; 40% less feed compared to AL) with standard (10%) or high (32%) protein (PRO) diets for 16-wks. This investigation was part of a larger study designed to assess the influence of protein source (soy vs. milk-based protein) on intestinal calcium absorption and bone (Gaffney-Stomberg et al., 2014). Only the rats consuming the milk-based protein diet were analyzed in the current study due to the amino acid content of the standard and high milk-based protein diets (Pasiakos and McClung, 2011). We wanted to test our hypothesis that dietary protein level modulates mTORC1 during sustained CR without protein quality (i.e., amino acid content) being a potential confounder. All study procedures were approved by the US Army Research Institute of Environmental Medicine Animal Care and Use Committee.

**Diet**

Purified study diets based on AIN-93 (Dyets, Inc., Bethlehem, PA) were modified to provide 10% and 32% protein to align with the lower and upper end of the current acceptable macronutrient distribution range (milk protein concentrate, Idaho Milk Products, Jerome, ID) (Gaffney-Stomberg et al., 2014). Milk protein concentrate was chosen because of its amino acid composition, digestion and absorption kinetics, and its effects on muscle intracellular signaling (Pasiakos, 2015). Chemical analysis of diets was performed to ensure nutrient content (Covance Laboratories, Dedham, MA; **Table 1**). The amount of feed provided to the CR rats was initially determined by averaging daily intake during the 14-d acclimation phase. Feed intake for CR fed rats was $16 \pm 2$ g·d$^{-1}$ whereas the AL rats consumed $26 \pm 3$ g·d$^{-1}$. Feed intake for the AL rats was assessed every 2 days such that adjustments could be made to ensure the CR rats maintained a 40% energy deficit.

**Body Mass and Composition**

Body mass was determined twice a week using a calibrated electronic scale (Ohaus, East Lyme, CT). Dual Energy X-ray Absorptiometry (DXA; Lunar iDXA, GE Lunar Corp., Madison, WI, USA) was used to assess body composition after the16 wk feeding intervention under anesthesia [1 mL·kg$^{-1}$ mixture of 40 mg·kg$^{-1}$ ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA), 10 mg·kg$^{-1}$ xylazine (Xyla-Ject; Phoenix Scientific, Inc., St. Joseph, MO), and 1.0 mg·kg$^{-1}$ acepromazine (Boehringer Ingelheim, St. Joseph, MO)]. Small animal software (enCore Version 11.40.004, 2007; GE Lunar Corp.) was used to determine body composition.

**Muscle Protein Content**

Whole gastrocnemius samples were homogenized under liquid nitrogen using a mortar and pestle. Sample was then placed in ice-cold homogenization buffer (1:10 wt/vol) containing 50 mM Tris–HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM DTT, 1 mM benz-amidine, 1 mM PMSF, 10 μg mL−1 trypsin inhibitor and 2 μg mL−1 aprotinin. Protein concentration of the homogenate was then determined using 660 nm Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). Homogenate total protein concentration was expressed relative to DXA derived fat-free mass to provide an estimate of muscle protein content (Thomson and Gordon, 2005;2006). The muscle protein content measure accounts for potential differences in water weight (Ianuzzo and Chen, 1979;Thomson and Gordon, 2006) and provides an assessment of protein content, allowing for an estimate of muscle growth, not whole-body fat-free mass, independent of water weight. Muscle protein content was calculated as:

Muscle protein content = protein concentration ($mg \cdot ml^{-1}$) x (homogenate vol (ml) ÷ muscle wt (mg)) x fat-free mass (g)

**Intracellular signaling**

Phosphorylation status and total protein expression of molecular markers associated with mTORC1 signaling were determined using Western blot. Homogenates used for muscle protein content estimations were centrifuged for 15 min at 10000 x g at 4°C, the supernatant (lysate) was collected and protein concentration analyzed (ThermoFisher). Muscle lysates were solubilized in Laemmli buffer, with equal amounts of total protein (10 µg) and separated by SDS-PAGE using precast Tris·HCl gels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to polyvinylidene fluoride membranes and exposed to commercially available primary antibodies specific to Akt, p-Akt$^{Ser473}$, mTOR, p-mTOR$^{Ser2448}$, p70S6K, p-p70S6K$^{Thr389}$, rpS6, p-rpS6$^{Ser235/236}$, AMPKα (Cell Signaling Technology, Danvers, MA, USA), and PGC-1α (Santa Cruz, Dallas, TX USA) at 4 °C overnight. Labeling was performed using secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology), and chemiluminescent reagent was applied (Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Blots were quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad). To confirm equal protein loading per well glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for mTORC1 associated proteins and heat shock protein 90 (HSP90) was used for AMPKα and PGC-1α. Phosphorylation and total protein were normalized to GAPDH or HSP90 and the ratio of phosphorylation-to-total protein was determined. All data are presented as fold change relative to AL 10% PRO.

**mRNA and miR expression**

mRNA expression of genes associated with intracellular regulation of muscle mass, including mTORC1 amino acid sensing proteins (Lars and Map4k3; TaqMan®), amino acid transporters (Slc38a2 and Slc7a5 TaqMan®), and energy utilization (Sirt1, Ppargc1a, Tfam, Ppara, Pparg; Bio-Rad), were determined using commercial available primers and in mixed gastrocnemius samples. miR that regulate targets in the mTORC1 pathway (hsa-miR-16-5p, hsa-miR-26b-5p, hsa-miR-99a-5p, hsa-miR-100-5p, hsa-miR-128a-3p, hsa-miR-133a-3p, hsa-miR-199a-3p, hsa-miR-221-3p) were analyzed using TaqMan® microRNA Assays (Applied Biosystems, Foster City, CA, USA). The roles of the mRNA and miR assessed are provided in **Supplemental Table 1**.

Total RNA was isolated in 20 mg muscle samples using a mirVana™ miRNA isolation kit (Invitrogen, Carlsbad, CA, USA); RNA quantity and quality were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA were synthesized into cDNA (iScript™ Advanced cDNA Synthesis Kit; Bio-Rad). miR were reverse-transcribed using the TaqMan® microRNA RT kit (Applied Biosystems) with the nine miR-specific stem-loop reverse transcript (RT) primers pooled in 1X-Tris-EDTA (TE) buffer for a final dilution of 0.05X for each miR RT primer. The RT primer pool (6 µl) was added to the RT reaction mix (0.3 µl 100mM dNTP, 3 µl enzyme, 1.5 µl 10× RT buffer, 0.19 µl RNase inhibitor) and 250 ng of total RNA (Le Carre et al., 2014). For both mRNA and miR, reverse transcription was conducted using a T100™ Thermal Cycler (Bio-Rad). RT-qPCR amplifications were conducted using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Samples were run in 20 µl reactions in triplicates, using iTaq™ Universal SYBR® Green Supermix

(Bio-Rad) of mRNA, and TaqMan® Univsersal PCR MasterMix (2X), no UNG (Applied Biosystems) for miR. All mRNA and miR were normalized to the geometric mean of U6 and B2M. Fold changes for mRNA and miR were calculated using the ΔΔ cycle threshold ($\Delta\Delta C_T$) method (Pfaffl, 2001) and expressed relative to AL 10% PRO.

**Citrate Synthase Activity Assay**

Citrate synthase activity was assessed using whole cell lysate from homogenized mixed gastrocnemius samples as described above. Enzyme activity was determined using a colorimetric assay analyzed on an ELx808 Absorbance Reader (BioTek®, Winooski, VT, USA), by combining 10 µl of diluted (1:10; 0.1 M Tris HCl pH 8.1) sample to 150 µl of reaction master mix (1 mL DNTB, 3 mg Acetyl CoA, and 8 mL 0.1 M Tris HCl pH 8.1). The reaction was initiated when 10 µl of 10 mM oxaloacetate was added to each well. Samples were read at 412 nm. Data were normalized to protein content.

**Plasma Amino acid concentrations**

Plasma amino acid concentrations were determined from blood collected by intra-cardiac puncture. Samples were tested in duplicate using HPLC and o-phthaldialdehyde post-column derivatization (Agilent 1100 Series HPLC; Agilent Technologies). Amino acid concentrations were used to determine the influence of dietary protein and energy status on mTORC1 signaling.

**Statistical Analysis**

A univariate ANOVA was conducted to determine the influence of energy status (AL vs. CR) and protein intake (10% vs. 32%) on body mass and composition, muscle protein content, Western blots, mRNA and miR expression, and serum BCAA. Bonferroni adjustments for multiple comparisons were performed if significant interactions (energy-

by-protein) were observed. Spearman's rank correlation coefficients were used to determine the relationship between muscle protein content, as determined by protein muscle content, and total Akt, mTOR, p70S6K, and rpS6. All mRNA and miR data were log transformed ($Log_2$) for statistical analysis, but presented as original values (median ± SEM). All other data presented as mean ± SEM. The α level for significances was set at $P < 0.05$. Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp. Armonk, NY).

**Results**

**Body mass, composition and muscle protein content**

Following the 16-wk feeding intervention a time-by-energy interaction was observed, change in total body and fat mass was lower ($P < 0.05$) in CR compared to AL fed rats, regardless of protein intake (**Fig. 1*A*-*B***). Fat-free mass increased ($P < 0.05$) from baseline to week 16 in all rats, with no effect of energy or protein intake (**Fig. 1*C***). Though fat-free mass was statistically similar between CR and AL at the conclusion of the 16-wk feeding intervention, muscle protein content was 38% lower (7 ± 2 g; $P < 0.05$) in CR than AL (**Fig. 1*D***).

**Anabolic intracellular signaling**

Phosphorylation status of $Akt^{Ser473}$, $mTOR^{Ser2448}$, $p70S6K^{Thr389}$, and $rpS6^{Ser235/236}$ were ($P < 0.05$) 1.72 ± 0.11, 1.41 ± 0.08, 1.95 ± 0.05, and 3.47 ± 0.05 fold lower, respectively, in CR compared to AL rats, regardless of protein intake (**Fig. 2*A***). Total protein of Akt, mTOR, p70S6K, and rpS6 were 1.85 ± 0.04, 1.71 ± 0.08, 2.23 ± 0.05, and 2.57 ± 0.05 fold lower, respectively, in CR compared to AL rats, with no effect of dietary protein intake (**Fig. 2*B***). Energy or dietary protein did not impact the ratio of phosphorylation-

to-total protein, except for an energy-by-protein interaction for rpS6, where CR 32% PRO was 1.98 ± 0.22 fold lower than AL 10% PRO (**Fig. 2*C***). Akt ($r$ = 0.43, $r^2$ = 0.18; **Fig. 3*A***) and rpS6 ($r$ = 0.64, $r^2$ = 0.36; **Fig 3*B***) were positively associated ($P < 0.05$) with protein content. No other associations were observed.

**mRNA and miR expression**

Energy and protein intake altered the expression of genes associated with amino acid sensing and transport upstream of mTORC1 (**Fig. 4*A*)**. Regardless of energy intake, amino acid transporter Slc38a2 expression was 1.19 ± 0.08 fold higher ($P < 0.05$) for 32% PRO versus 10% PRO. No effect of energy or protein intake was observed for Slc7a5. Expression of amino acid sensing Map4k3 and Lars were 1.28 ± 0.06 and 1.28 ± 0.08 fold lower ($P < 0.05$) for CR than AL, independent of dietary protein. No effects of energy or protein intake were observed in the expression of any miR (**Fig. 4*B***).

**Energy Utilization**

Regardless of protein intake, Ppargc1a expression was 1.74 ± 0.20 fold higher ($P < 0.05$) in CR compared to AL (**Fig. 5*A***). No effect of energy or protein was observed for gene expression of Sirt1, Tfam, Ppara, and Pparg. Total protein for master regulators of energy utilization, AMPKα and PGC-1α, were similar between AL and CR (**Fig. 5*B***). Additionally, citrate synthase activity was also similar between CR and AL (**Fig. 5*C***). No effect of protein was observed for AMPKα PGC-1α protein expressoin and citrate synthase activity.

**Plasma branched-chain amino acid profile**

A main effect of energy was observed for plasma branched-chain amino acids; leucine, isoleucine and valine concentrations were 25.9 ± 5.0 μmol·L$^{-1}$, 19.2 ± 3.1 μmol·L$^{-1}$ and

27.3 ± 5.5 μmol·L$^{-1}$ lower ($P < 0.05$) in CR than AL (**Table 3**). A main effect of protein was also observed for branched-chain amino acids; leucine, isoleucine and valine concentrations were 12.5 ± 5.0 μmol·L$^{-1}$, 6.6 ± 3.1 μmol·L$^{-1}$ and 19.6 ± 5.5 μmol·L$^{-1}$ higher ($P < 0.05$) in rats consuming 32% PRO versus 10% PRO. No energy-by-protein interactions were observed.

**Discussion**

The primary findings from this investigation were that prolonged CR (16-wks; 40% total energy requirements) led to downregulation of fasting mTORC1 signaling activity and inhibition of protein translation and phosphorylation (i.e., activity) of Akt, mTOR, p70S6K, and rpS6 under fasted conditions. The systematic downregulation of mTORC1 associated protein expression and activity, particularly the decline rpS6, was associated with diminished muscle protein content. These findings link the molecular regulation of mRNA translation initiation and, possibly MPS, with a measure of long-term muscle protein status in response to underfeeding. Contrary to our hypothesis, the high protein diet, fed as a percentage of total calorie intake, did not attenuate declines in mTORC1 signaling and muscle protein content compared to standard protein intake. Furthermore, the inhibition of mTORC1 associated protein translation during CR did not appear to be regulated by miR. Overall, findings from this study indicate that prolonged CR alters protein translation and downregulates mTORC1 activity, independent of associated miR. Moreover, CR appears to override the protein synthetic stimulus of increased dietary protein intake, resulting in diminished muscle protein content.

The degree to which CR alters protein metabolism appears to be dependent on the magnitude and duration of the restriction (Pasiakos et al., 2015). In short-term human

studies, fasting and postprandial MPS rates are downregulated within 5-10 days of 40% CR (Pasiakos et al., 2010;Areta et al., 2014;Hector et al., 2015;Murphy et al., 2015). Consuming a high protein diet, particularly high-quality protein-containing meals, attenuates these declines and restores fasting and postprandial MPS rates to levels observed during energy balance (Pasiakos et al., 2013;Areta et al., 2014). However, these effects of short-term CR and dietary protein manipulations on MPS were not reflected in concurrent changes in mTORC1 signaling. Interestingly, Miller et al., (Miller et al., 2013) observed a tissue specific decline in mTORC1 signaling, as rpS6 phosphorylation and total protein were lower in both heart and liver, but not skeletal muscle after 6-wks of CR (40% total energy requirements). In the present 16-wk study, we observed that a 40% CR downregulated skeletal mTORC1 protein translation and phosphorylation, regardless of dietary protein intake. These findings suggest a time-dependent adaptation to the mTORC1 signaling pathway in response to underfeeding. More specifically, short-term CR may induce (5-10 days, 40% restriction) reductions in MPS to conserve energy and substrate availability, but this does not elicit concomitant adaptations in mTORC1. Under these circumstances, it appears that consuming higher levels of protein may saturate basal energy and whole-body protein requirements, and provide additional substrate to maintain MPS (Pasiakos et al., 2013). However, as the duration of CR is extended, the anabolic potential of skeletal muscle (and other tissues) appears to diminish. This inhibition of mTORC1 signaling and maintenance of the translation of AMPKα and PGC-1α, key regulators of energy utilization, is in agreement with Miller et al., (Miller et al., 2012;Miller et al., 2013) who suggested that selective

translation of key proteins during CR maintains pathways required to preserve cellular function at the expense of anabolic processes.

Estimates of muscle protein content were lower for CR than AL, and positively associated with mTORC1 signaling. Downregulated expression of rpS6, the downstream target of mTORC1 that triggers translation initiation and cellular growth (Magnuson et al., 2012), explained 36% of the variability in muscle protein content after the 16-wk intervention, with lower total rpS6 indicative of lower muscle protein content. AL and CR fed rats both gained body weight during the intervention, albeit the rate at which the CR rats grew was attenuated (Gaffney-Stomberg et al., 2014). Interestingly, the extra total mass gained for the AL rats was nearly all body fat, as fat-free mass by DXA was statistically similar between AL and CR. However, CR fed rats accrued 38% less muscle protein than AL fed rats during the 16-wk intervention, suggesting DXA may not be the best tool for estimating muscle protein mass in this model. We also recognize that not examining muscle mass or cross-sectional area could be considered a limitation, unfortunately at the time of sacrifice the gastrocnemius was not weighed nor were the tissue samples not fixed for histology at the time of collection. Muscle mass and cross-sectional area may have provided additional information regarding the role of altered mTORC1 signaling on muscle protein content at the level of skeletal muscle during prolonged CR. Furthermore, having mixed gastrocnemius muscle mass from 12-wk old Sprague Dawley rats would have been beneficial to use a baseline values and calculate difference in skeletal muscle mass following the 16-wk feeding intervention. Regardless, the observation that mTORC1 signaling was highly correlated with muscle protein

content suggests that mTORC1 responses to underfeeding are largely responsible for diminished protein content.

Increased extracellular amino acid concentrations, particularly leucine, upregulates amino acid transporter expression and uptake of amino acids into the intracellular amino acid pool and produces a robust increase in mTORC1 activity (Drummond et al., 2010;Churchward-Venne et al., 2014). The high protein diets in this study expanded the extracellular branched-chain amino acid pools, but failed to modulate the expression and activity of the mTORC1 pathway. We suspect that ad libitum access to feed contributed to this null effect. Extracellular leucine concentrations must surpass a certain threshold to optimally increase cellular uptake and stimulate mTORC1 (Moore et al., 2009;Norton et al., 2009;West et al., 2011). Providing free access to feed diminished the likelihood that protein consumed at any one point during the day surpassed the leucine threshold (Norton et al., 2009). Consistent with this explanation, branched-chain amino acid levels for the high protein CR fed rats were lower than AL 10% PRO fed rats. The lower circulating BCAA were observed despite the fact that CR 32% PRO fed rats consumed twice (4.9 $g \cdot d^{-1}$) the amount of dietary protein per day than AL 10% PRO (2.4 $g \cdot d^{-1}$). Furthermore, that no physiologically relevant ($\geq 2$ fold) increase in expression of the amino acid transporters Slc38a2 and Slc7a5 occurred with concomitant CR-induced reductions in the expression of amino acid sensing genes Map4k3 and Lars supports the theory that extracellular leucine availability was never optimal. Protein may have been stimulatory, despite CR, if food was provided at discrete times and had sufficient protein to adequately increase plasma amino acid levels.

To the best of our knowledge this is the first investigation to assess the role of skeletal muscle miR expression on mTORC1 signaling following adaptations to prolonged CR. Recent studies have indicated miR expression play a role in the regulation of muscle mass (McCarthy and Esser, 2007), via the mTORC1 pathway (Jin et al., 2013;Wei et al., 2013;Jia et al., 2014;Zacharewicz et al., 2014). In contrast, miR in the current investigation were not impacted by CR and had no effect on mTORC1. Using a targeted assessment of specific miR in the current study may have limited our ability to capture altered expression other potentially relevant miR that could have been measured using a microarray or miR-seq. However, a global approach was not taken because our intent was to determine if miR previously shown to regulate specific mTORC1 associated proteins were also influenced by CR or high protein intake. Findings from the present investigation indicate that alterations of mTORC1 signaling following sustained CR are not regulated by the expression of miR-99a or miR-100-5p.

In conclusion, data from this study provide a link between mTORC1 signaling, muscle protein content, and prolonged underfeeding. The systematic downregulation of mTORC1 signaling activity with CR, regardless of protein intake, was likely the result of inhibited protein translation and subsequently lower phosphorylation of Akt, mTOR, p70S6K, and rpS6. Furthermore, while the effects of CR on mTORC1 were clearly demonstrated, they appear to be independent of miR expression. These data also demonstrate that high dietary protein intake alone, when provided as a percentage of total energy and evenly distributed in food consumed ad libitum, is not sufficient to attenuate mTORC1 adaptations to prolonged CR.

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**Conflict of Interest Statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions**

JPM and SMP: Study design, oversight, and sample collection. LMM, DAR, MB, YE: Completed experiments and analyzed and managed data. LMM, DAR, AJY, JPM, RAF, SPM: Data interpretation and manuscript preparation. Reviewed by all authors.

**Abbreviations**

Akt: protein kinase B

AL: ad libitum

AMPKα: AMP-activated protein kinase α

B2M: Beta-2-Microglobulin

CR: calorie restriction

DXA: dual energy X-ray absorptiometry

GAPDH: glyceraldehyde-3 phosphate dehydrogenase

Lars: Leucyl-tRNA Synthetase

Map4k3: Mitogen-Activated Protein Kinase Kinase Kinase Kinase 3

miR: microRNA

MPS: muscle protein synthesis

mTORC1: mechanistic target of rapamycin complex 1

PGC-1α: Peroxisome proliferator-activated receptor gamma, coactivator 1, alpha

Ppara: Peroxisome proliferator-activated receptor alpha

Pparg: Peroxisome proliferator-activated receptor gamma

PRO: protein

p70S6K: 70 kDa S6 kinase

RDA: recommended dietary allowance

rpS6: ribosomal protein S6

Sirt1: Sirtuin 1

Slc38a2: Solute Carrier Family 38, Member 2

Slc7a5: Solute Carrier Family 7, Amino Acid Transporter Light Chain, L System

U6: RNA, U6 Small Nuclear 1

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**Figure Legends**

**Fig 1.** Mean (± SEM) [*n* = 10 (AL 10% PRO; ■), *n* = 10 (AL 32% PRO; ■), *n* = 10 (CR 10% PRO; ■), *n* = 10 (CR 32% PRO; □)] body mass (**A**), fat mass (**B**), fat-free mass (**C**), and muscle protein content (**D**). Data analyzed using a univariate ANOVA with Bonferroni correction to determine main effects of energy (AL vs. CR), protein (10% vs. 32%) and energy-by-time interactions. †Week 16 different from Baseline; $P < 0.05$. +Time-by-energy interaction, CR different from AL at Week 16; $P < 0.05$. *CR different from AL; $P < 0.05$.

**Fig 2.** Mean (± SEM) [*n* = 10 (AL 10% PRO), *n* = 10 (AL 32% PRO), *n* = 10 (CR 10% PRO), *n* = 10 (CR 32% PRO)] phosphorylation (**A**) and totals (**B**) relative to GAPDH, and phosphorylation relative to total (**C**). Data analyzed using a univariate ANOVA with Bonferroni correction to determine main effects of energy (AL vs. CR), protein (10% vs. 32%) and energy-by-protein interactions. *CR body mass different from AL; $P < 0.05$.+Energy-by-protein interaction, CR 32% PRO different than AL 32% PRO; $P < 0.05$.

**Fig 3.** Association of total Akt (**A**) and rpS6 (**B**) to muscle protein content [*n* = 10 (AL 10% PRO ♦), *n* = 10 (AL 32% PRO ■), *n* = 10 (CR 10% PRO ▲), *n* = 10 (CR 32% PRO ○)]. Data analyzed using a spearman rho correlation coefficient. Significant associations were determined as $P < 0.05$.

**Fig 4.** Median (± SEM) [*n* = 10 (AL 10% PRO; ■), *n* = 10 (AL 32% PRO; ■), *n* = 10 (CR 10% PRO; ■), *n* = 10 (CR 32% PRO; □)] mRNA (**A**) and miR (**B**) expression. Data

analyzed using a univariate ANOVA with Bonferroni correction to determine main effects of energy (AL vs. CR), protein (10% vs. 32%) and energy-by-protein interactions. *CR body mass different from AL; $P < 0.05$.+32% PRO different than 10%; $P < 0.05$.

**Fig 5.** Median (± SEM) [$n = 10$ (AL 10% PRO; ■), $n = 10$ (AL 32% PRO; ■), $n = 10$ (CR 10% PRO; ■), $n = 10$ (CR 32% PRO; □)] mRNA (**A**) and mean (± SEM) total AMPKα and PGC-1α (**B**) expression and citrate synthase activity (**C**). Data analyzed using a univariate ANOVA with Bonferroni correction to determine main effects of energy (AL vs. CR), protein (10% vs. 32%) and energy-by-protein interactions. *CR different from AL; $P < 0.05$.

**Table 1.** Energy and macronutrient composition of experimental diets[1]

| | **AL 10% PRO** | **AL 32% PRO** | **CR 10% PRO** | **CR 32% PRO** |
|---|---|---|---|---|
| Energy (kcal·d$^{-1}$) | 95.1 | 96.7 | 57.1 | 58.0 |
| Protein (g·d$^{-1}$) | 2.4 | 8.2 | 1.4 | 4.9 |
| BCAA (mg·d$^{-1}$)[2] | | | | |
| Leucine | 242.0 | 900.0 | 145.2 | 540.0 |
| Isoleucine | 127.5 | 482.5 | 76.5 | 289.5 |
| Valine | 145.0 | 550.0 | 87.0 | 330.0 |
| Carbohydrate (g·d$^{-1}$) | 17.1 | 10.9 | 10.3 | 6.7 |
| Fat (g·d$^{-1}$) | 1.9 | 2.3 | 1.1 | 1.4 |

[1]Diets formulated based on AIN-93 with *Ad Libitum* fed rats consuming 25 g·d$^{-1}$ and Calorie Restricted fed rats consuming 15 g·d$^{-1}$. [2]BCAA; branched-chain amino acid

**Table 2.** Branched-chain amino acids profiles following 16-week feeding intervention

| **Amino Acids** | | | | | ***P* Value** | | |
|---|---|---|---|---|---|---|---|
| **(μmol·L$^{-1}$)** | **AL 10% PRO** | **AL 32% PRO** | **CR 10% PRO** | **CR 32% PRO** | **Energy** | **Protein** | **E x P[2]** |
| **BCAA[1]** | 442.5 ± 7.4 | 472.6 ± 17.8 | 361.5 ± 14.9 | 408.8 ± 10.7 | < 0.01 | < 0.01 | 0.52 |
| **Leucine** | 154.0 ± 3.8 | 166.7 ± 6.6 | 128.3 ± 5.5 | 140.6 ± 3.9 | < 0.01 | 0.02 | 0.97 |
| **Isoleucine** | 93.5 ± 1.8 | 96.9 ± 3.9 | 71.1 ± 3.8 | 80.9 ± 2.5 | < 0.01 | 0.04 | 0.31 |
| **Valine** | 195.0 ± 3.0 | 209.0 ± 7.5 | 162.1 ± 5.8 | 187.3 ± 4.9 | < 0.01 | < 0.01 | 0.32 |

Values mean ± SEM. [1]BCAA; branched-chain amino acids. [2]E x P; energy-by-protein interaction

**Figure 1.** Change in body mass and composition

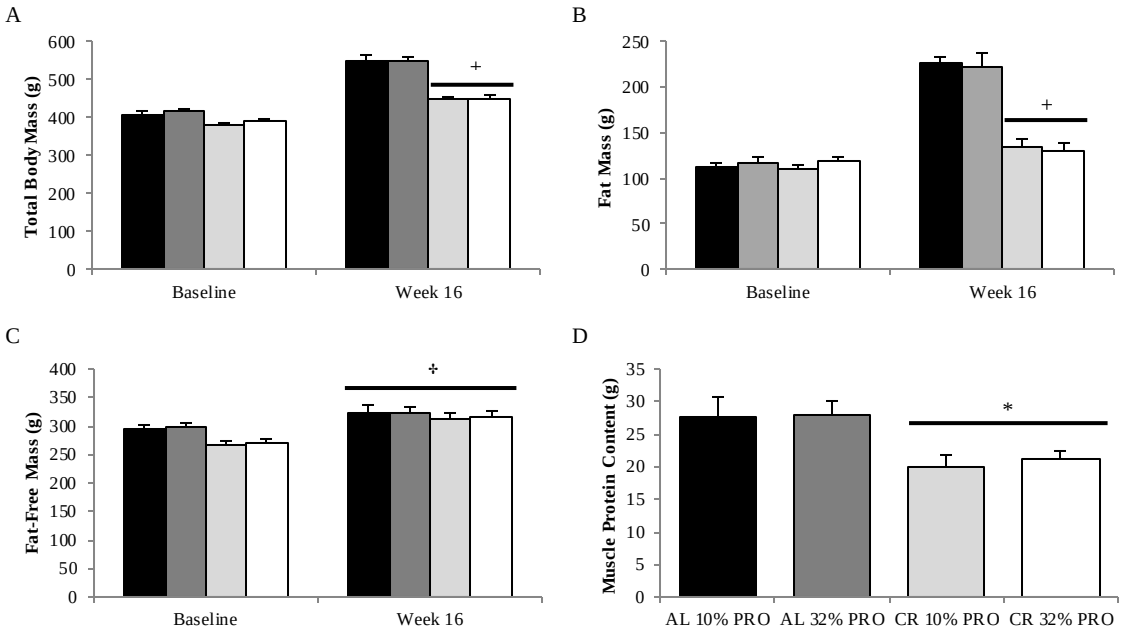


**Figure 2.** mTORC1 signaling

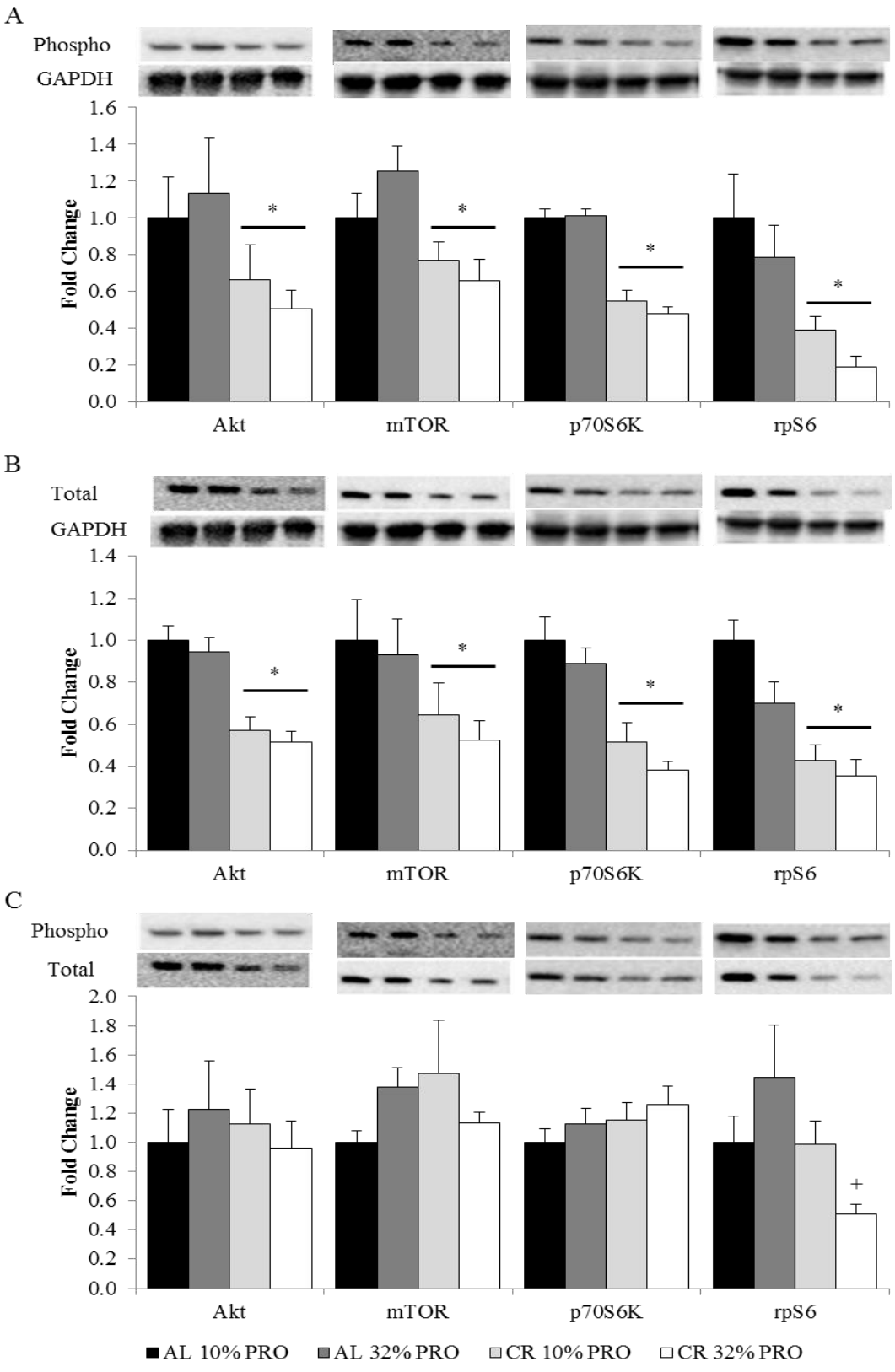


**Figure 3.** Correlation anabolic signaling and muscle protein content

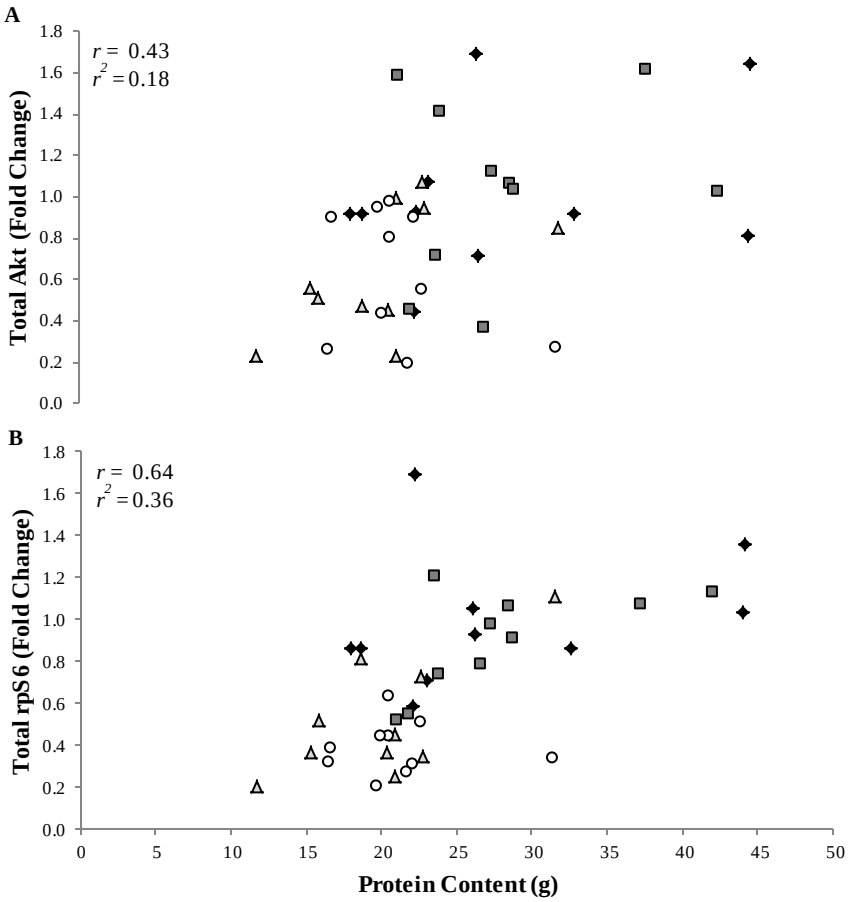


**Figure 4.** mRNA and miR expression

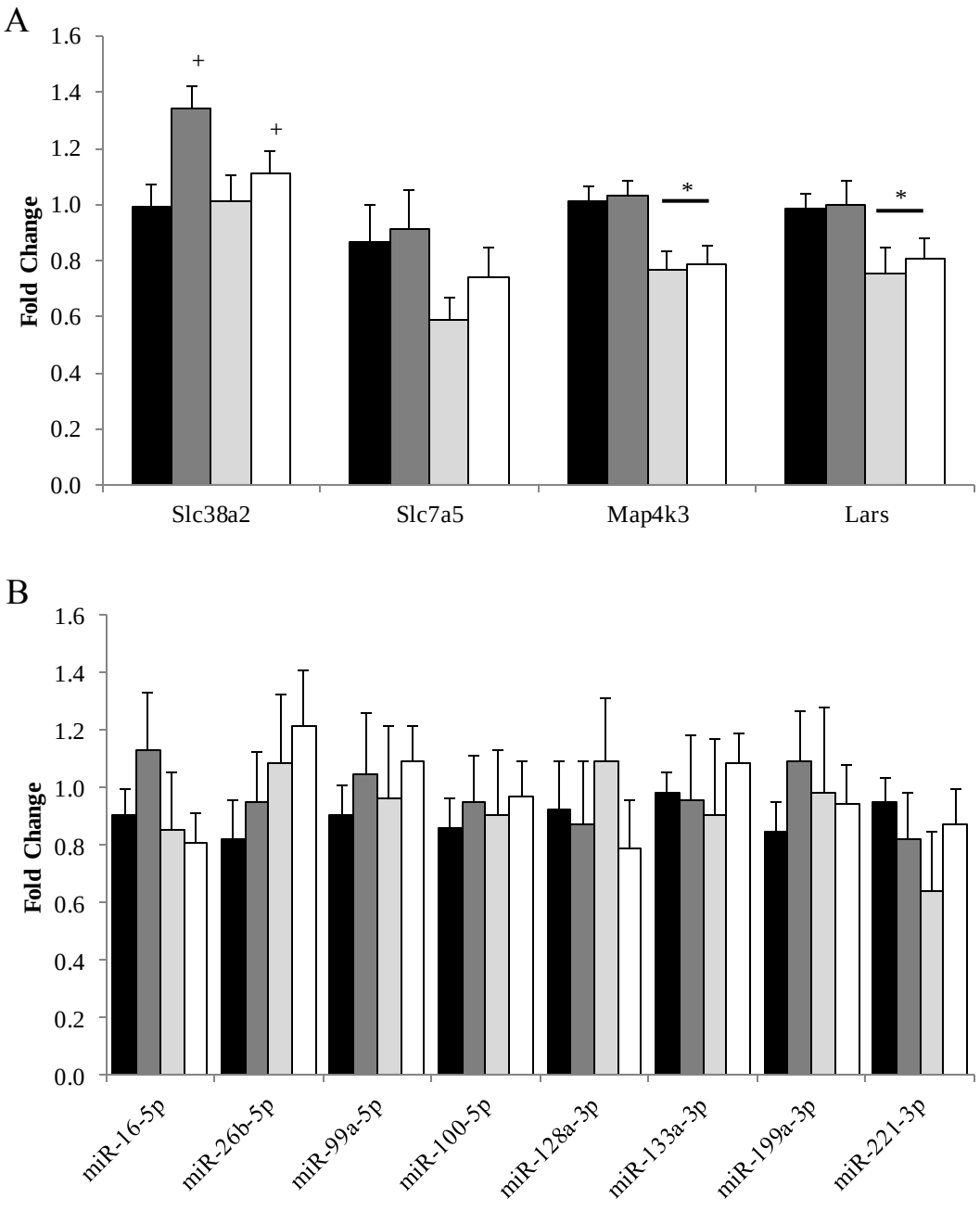
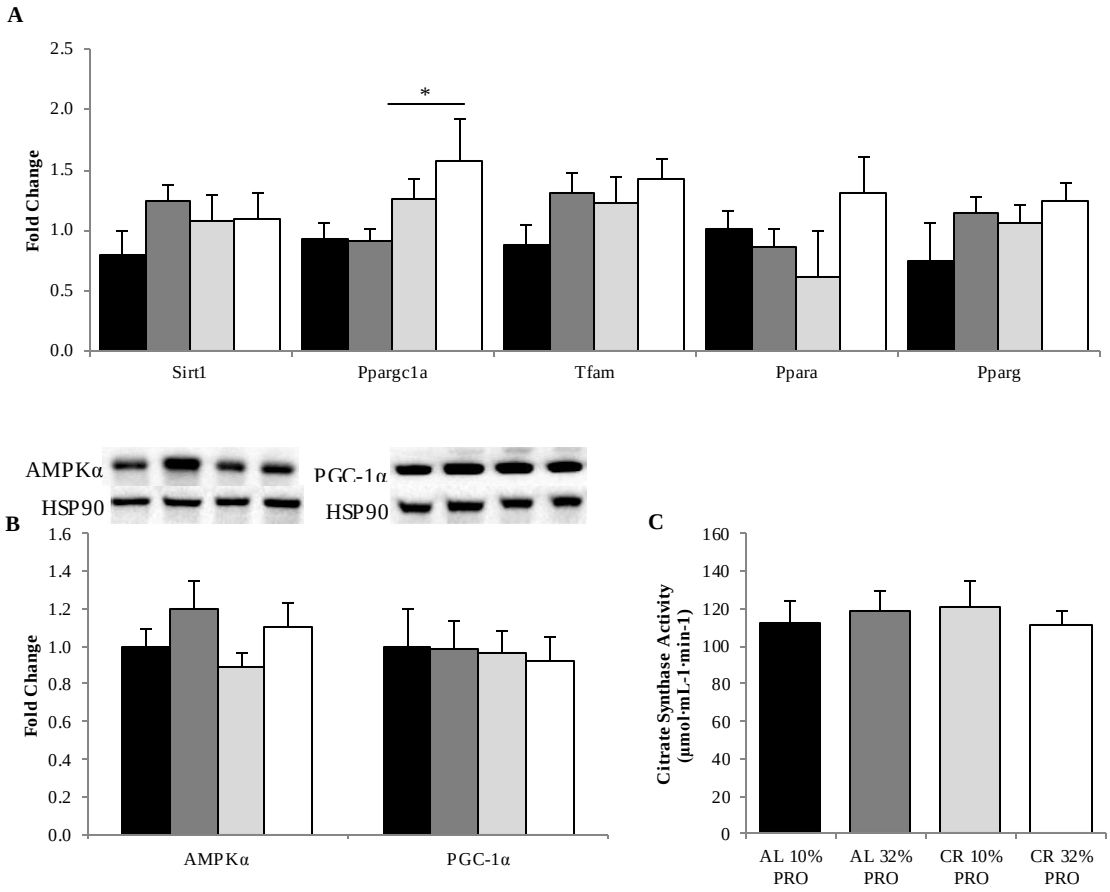


**Figure 5.** Energy utilization



**Supplemental Table 1.** mRNA and miR targets and function

| Name | Function |
|---|---|
| ***mRNA*** | |
| Slc38a2 | Transporter; sodium dependent cellular uptake of amino acid |
| Slc7a5 | Transporter; cellular uptake of large neutral amino acids |
| Map4k3 | Amino acid sensor stimulating activation of p70S6K |
| Lars | Leucine sensor interacting directly with Rag GTPase to active mTORC1 |
| Sirt1 | NAD-dependent protein deacetylase contributing to regulation of energy utilization |
| Ppargc1a | Transcription factor regulating genes involved in mitochondrial biogenesis, lipid metabolism and carbohydrate metabolism |
| Tfam | Mitochondrial transcription factor, promotes mitochondrial biogenesis |
| Ppara | Transcription factor regulating fatty acid uptake and oxidation |
| Pparg | Transcription factor regulating fatty acid storage |
| ***miR*** | |
| miR-16-5p | Inhibits IGF-1R and regulates cell proliferations |
| miR-99a-5p | Inhibits gene expression and protein translation of mTOR |
| miR-100-5p | Inhibits gene expression and protein translation of mTOR and Raptor |
| miR-128a3p | Inhibits phosphorylation of Akt |
| miR-133a-3p | Inhibits protein expression of IGF-1R and phosphorylation of Akt |
| miR-199a-3p | Inhibits gene expression of mTOR |
| miR-221-3p | Stimulates protein translation of Akt through inhibition of PTEN |

Slc38a2: Solute Carrier Family 38, Member 2; Slc7a5: Solute Carrier Family 7, Amino Acid Transporter Light Chain, L System; Map4k3: Mitogen-Activated Protein Kinase Kinase Kinase Kinase 3; Lars: Leucyl-tRNA Synthetase; Sirt1: Sirtuin 1; Ppargc1a: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Tfam:

mitochondria transcription factor; Ppara: Peroxisome proliferator-activated receptor alpha; Pparg: Peroxisome proliferator-activated receptor gamma.

# V. CONCLUSION

Primary findings from this series of investigations provide preliminary evidence that c-miRNA profiles are altered under conditions that modulate skeletal muscle anabolism. Specifically, results from this work identify c-miRNA as predictive markers for aging, as well as the anabolic response to an acute bout of resistance exercise and alterations in protein synthesis following short-term CR. Our initial investigation demonstrated that miR-19b-3p, miR-206 and miR-486 distinguished between younger and older male participants, and were correlated with age-associated alterations in body composition and metabolic health. Integrative bioinformatic analysis determined mutual pathways of these 3 discriminate miRNA were identified as FoxO signaling, p53 signaling, cell cycling, PI3K-Akt signaling, apoptosis, and AMPK signaling, revealing the potential for these miRNA to be regulators of skeletal muscle anabolism and atrophy, metabolism, immune-regulation, and oxidative stress. Together, these comprehensive analyses identified miR-19b-3p, miR-206 and miR-486 as predictive of aging, and illustrate that common molecular pathways may have a critical role in regulation of age-associated changes in body composition and metabolic health.

Following acute resistance exercise, using principle component analysis (PCA) and Ingenuity Pathway Analysis (IPA) we found that divergent c-miRNA expression with aging was predictive of 'anabolic resistance,' as IGF-1 and mTOR signaling cascades were identified as top canonical pathways in younger but not older males. These findings were supported by activation (e.g., phosphorylation) status of mTORC1 signaling in skeletal muscle following resistance exercise. Western blotting results determined that younger participants experienced an upregulation ($P < 0.05$) in upstream (p-Akt$^{Ser473}$) and

downstream (p-70S6K1$^{Thr389}$) targets of mTORC1, while no change was observed in older participants following acute resistance exercise (1). Comparing skeletal muscle Western blot data to expression of the top 10 c-miRNA identified by PCA, positive associations ($P < 0.05$) to both p-Akt$^{Ser473}$ and p-70S6K1$^{Thr389}$ were observed to the expression of miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, and miR-195-5p. Though several previous investigations have examined the influence of aging on c-miRNA expression date (2-6), findings from the present investigation are the first to examine their use as a noninvasive predictive marker of the acute adaptive response following resistance exercise. Coupling traditional bench top techniques with newer integrative analytic techniques assists in the validation of IPA findings. Furthermore, these findings indicate c-miRNA may be used as noninvasive markers to predict adaptations reflective of molecular processes in skeletal muscle to acute resistance exercise with aging.

Calorie restriction-induced weight loss not only results in desirable losses in fat mass, but can also lead to undesirable reductions in skeletal muscle mass (7). In an older population who may already be experiencing age-associated declines in muscle mass, weight loss regimens are feared to further compromise physical function and increase frailty (8). However, as 35% of adults 60 yrs and older are considered obese there is an increased need for this population to engage in weight loss interventions to improve metabolic health (9). As such, there is a continued need to understand potential consequences to skeletal muscle physiology during CR in older adults to determine appropriate exercise and nutritional countermeasures.

In our second intervention CR resulted in an upregulation ($P < 0.05$) in c-myomiR scores (median fold change of miR-1-3p, miR-133a-3p, miR-133b, and miR-206), with significant increases in the expression of miR-133a and miR-133b compared to weight maintenance values. Though not statistically significant expression of miR-1-3p tended ($P = 0.07$) to be greater during CR, while no effect of CR was observed miR-206 expression. Additionally, following the 30% 28-d CR period an inverse association ($P < 0.05$) was observed with c-myomiR scores and whole-body protein synthesis ($r = -0.70$, $r^2 = -0.51$), with greater expression of c-myomiR reflective of lower rates of protein synthesis.

In agreement with findings from the human trial, *in vitro* analysis determined that expression of c-myomiR in the media of C2C12 myotubes were increased ($P < 0.05$) with a downregulation in the of skeletal muscle protein synthetic rate in CR compared to control cells. While myomiR are highly expressed in skeletal muscle, comprising nearly 25% of all miR within the tissue (10), miR-1-3p, miR-133a-3p and miR-133b are present in other tissue, such as heart and bone. Corroboration of findings from the human trial by myotubes in our cell culture experiment provide further evidence that alterations in c-myomiR by CR are reflective of skeletal muscle. Together, findings from *in vivo* and *in vitro* analysis suggest that lower rates of protein synthesis following CR are reflected by an upregulation in expression of myomiRs.

Interestingly, in our initial investigation, comparison of c-myomiR with aging under resting fasted conditions revealed that expression miR-1-3p, miR-133a-3p, and miR-206 were all $\geq 1.5$ fold higher in older compared to younger participants. Furthermore, an inverse correlation was observed with miR-133a-3p ($r = -0.61$) and miR-206 ($r = -0.51$)

expression compared to percent fat-free mass. Unfortunately the second investigation is limited by the fact that no assessment of body composition was conducted. Whether elevated expression in c-myomiR correlate with declines in fat-free mass following the 30% 28-d CR cannot be determined. Regardless, combining results from our first and second investigation suggests that aging results in an upregulation of c-myomiR that may be further exacerbated by short-term CR. Future investigations must include measurements of body composition to determine if alterations in c-myomiR reflect changes in fat-free mass. Additionally, while our investigation established an association between c-myomiR expression and whole-body protein synthesis, it is important to note that skeletal muscle only contributes 30-45% whole-body protein turnover (11). As such, future investigations should conduct measurements of c-myomiR expression with assessment of skeletal muscle protein synthesis to determine if c-myomiR reflects alterations in protein synthesis at the skeletal muscle level.

Contrary to the observation that short-term CR upregulated c-myomiR, following a prolonged (16-wk) 40% CR period in Sprague Dawley rats no alteration in expression miRNA was observed within skeletal muscle. Independent of dietary protein intake, phosphorylation status of mTORC1 related signaling proteins were downregulated ($P < 0.05$) due to reduction in total protein of Akt, mTOR, p70S6K, and rpS6 in CR compared to *ad libitum* (AL) fed rats. Additionally, Akt ($r = 0.43$, $r^2 = 0.18$) and rpS6 ($r = 0.64$, $r^2 = 0.36$) were positively associated ($P < 0.05$) with muscle protein content. Despite these translational modifications observed in the mTORC1 pathway, there was no change in the expression of associated miRNA. Previous investigations (12-14) have reported that overexpressing members of the miR-99/100 family inhibits gene expression and protein

translation of mTORC1 signaling proteins, downregulating the activity of the pathway and ultimately cellular proliferation. Findings from our investigation suggest that downregulation in mTORC1 within skeletal muscle following CR may occur independently of miRNA expression. Conversely, it is possible that modification in the expression of miRNA that resulted in the CR-induced translational modifications to mTORC1 signaling occurred during the initial phases of CR. To determine if miRNA are a potential regulatory mechanism modifying mTORC1 signaling with CR, a time course experiment should be conducted assessing miRNA expression at initiation (3-7 days), short-term (3 wks) and prolonged (16-wk) CR.

Despite a lack of effect on miRNA expression, this investigation is the first to show a systematic decline in mTORC1 signaling within skeletal muscle following prolonged CR. Previously, a tissue specific decline in mTORC1 signaling has been reported, with rpS6 phosphorylation and total protein lower in both heart and liver, but not skeletal muscle after 6-wks of CR (40% total energy requirements) (15). In the present 16-wk study, we observed that a 40% CR downregulated skeletal mTORC1 protein translation and phosphorylation, regardless of dietary protein intake. These findings suggest that a time-dependent adaptation to the mTORC1 signaling pathway in response to underfeeding. Additionally, while CR inhibited mTORC1 signaling, energy utilization pathways were maintained, as no effect of energy status was observed for AMPK$\alpha$ and PGC-1$\alpha$ total protein or citrate synthase activity. Maintenance of key energy utilization regulators suggests that selective translation of key proteins during CR maintains pathways required to preserve cellular function at the expense of anabolic processes.

High protein CR diets have previously been shown to preserve anabolic sensitivity to a protein-containing meal, and spare skeletal muscle mass during short-term CR (16-19). Findings from our investigation show no effect of dietary protein on maintenance of mTORC1 signaling with prolonged CR, despite high protein diets expanding the extracellular branched-chain amino acid pools. Recent investigations have shown that daily distribution of dietary protein intake can influence rate of protein synthesis during weight maintenance and calorie restriction (19, 20). Daily distribution likely impacts protein synthesis due to the fact that extracellular leucine concentrations must surpass a certain level to optimally increase cellular uptake and stimulate mTORC1 (21-23). Providing rats free access to feed diminished the likelihood that protein consumed at any one point during the day surpassed the leucine threshold (21). Protein may have been stimulatory, despite CR, if food was provided at discrete times.

Overall, findings from this series of investigations provide new insight into alterations of c-miRNA with aging, acute resistance exercise and CR. Suggestion that c-miRNA participate in cell-to-cell communication indicates they have an endocrine-like function (24). Having a functional role on alterations in molecular pathways within tissue indicates c-miRNA may offer a unique perspective as noninvasive markers of physiological adaptions to aging, exercise and dietary intervention. However, the field of c-miRNA research is relatively young, having only existed for the last 10 years. As this area of research grows, advancement of methodologies to isolate exosomes and microvesicles, as well as determination of tissue source *in vitro* will be required to fully realize the functional potential of c-miRNA to determine causal effects (25). Data from our initial two investigations provide preliminary evidence that altered c-miRNA profiles

reflect acute exercise-induced adaptions within skeletal muscle and rate of protein synthesis following CR. Despite c-miRNA reflecting rates of protein synthesis in our human and cell experiments, miRNA expression was not altered with downregulation in mTORC1 signaling following prolonged CR. Discrepancies between investigations may be due to miRNA being measured after different lengths of CR. A time course experiment should be conducted to determine if length of CR influence expression of miRNA in circulation and skeletal muscle. Regardless, findings from this series of investigations provide preliminary evidence that c-miRNA profiles are altered under conditions that modulate skeletal muscle anabolism. Future investigations should aim to assess c-miRNA in skeletal muscle derived exosomes and microvesicles to determine the functional implication of these altered c-miRNA profiles.

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