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Impacts of microRNAs on Skeletal Muscle
Protein Synthesis and Mitochondrial Quality

Impacts of microRNAs on Skeletal Muscle
Protein Synthesis and Mitochondrial Quality

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Kinesiology

by

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University of Arkansas
Bachelor of Science in Kinesiology, 2012

May 2015
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This thesis is approved for recommendation to the Graduate Council.

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Abstract

microRNA (miRNA) post-transcriptional modification is becoming a well-established mechanism for controlling mRNA translation. microRNAs -1, -133, and -206 are under the control of skeletal muscle promoters and affect muscle plasticity and metabolic health. A detailed review on the generation and processing of miRNAs with a view to skeletal muscle brings up intriguing connections in the transcriptional connections between multiple miRNAs. Additionally, exciting new research has defined a role of miRNAs in skeletal muscle mitochondria showing an additional, direct link to metabolic function. Multiple investigations in models of exercise, aging, hypertrophy, and injury have shown how these interventions can affect miRNA content and activity. Because skeletal muscle is such an abundant and metabolically active tissue, it is important to understand its detailed physiology. Individual overexpression of miR -1 resulted in ~25% less phosphorylation of Akt and phosphorylation of p70S6K1 but did not change overall basal protein synthesis. These same measures of protein synthetic signaling were unaltered in cells overexpressing miR-133b. Additionally, gene and protein contents of COX-IV, a surrogate measure of mitochondrial content, were higher in both miR-1 (~100%) and -133b (~45%) overexpressed cells. Additionally, any change in mitochondrial content was separate from TFAM or PGC-1α protein as neither was changed by miR-1 or -133 overexpression. Together, these results indicate a clear advancement in the knowledge of the effects miRs -1 and -133 have on protein synthesis and on the mitochondrial network by taking clear steps towards understanding the effects of miRs -1 and -133b on protein synthesis and the mitochondrial network through assessment of mitochondrial content and turnover.

Acknowledgments

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I. Introduction

Since their initial discovery as temporal controllers of developmental stages in Ceanorhabditis elegans in 1993 (R. C. Lee, Feinbaum, & Ambros), the knowledge base surrounding microRNAs (miRNAs) has grown substantially. The research into these important modulators of protein expression still creates more questions than are answered. Though much elaboration is still needed, miRNAs have been connected to many disease states including cancers and chronic diseases (Guay, Roggli, Nesca, Jacovetti, & Regazzi, 2011).

Metabolic diseases such as diabetes are the result of whole body insulin resistance, which begins with skeletal muscle insulin resistance (DeFronzo & Tripathy, 2009). Lifestyle conveniences and sedentary behavior have greatly increased the prevalence of obesity and diabetes (Centers for Disease Control, 2013). Diabetic skeletal muscle has been shown to be resistant to the anabolic effects of exercise both by displaying abnormal protein turnover and impaired mitochondrial growth (Nilsson et al., 2013; Nilsson et al., 2010). These deficiencies can exacerbate the insulin resistant phenotype. Because of their common link over metabolic control, scientists need to focus their attention to miRNAs in skeletal muscle.

A subset of miRNAs that are specifically expressed in skeletal muscle (termed myomiRs) have large implications for controlling metabolic activity and thus health of an entire organism (Guay et al., 2011; Lagos-Quintana et al., 2002). I have previously observed that miRNAs -1 and -133b are altered in obesity/diabetes and respond differentially to resistance exercise (unpublished observations). miRNA-1 has been highly linked to muscle growth through targeting of Insulin-like Growth Factor 1 (IGF-1) and its activity on the Protein Kinase B

(Akt)/mammalian Target of Rapapmycin (mTOR) pathway of protein synthesis (Elia et al., 2009). To date, this has been limited to investigations in pathological cardiac hypertrophy (Elia et al., 2009; Feng, Chen, George, Feng, & Chakrabarti, 2010). miRNA-133b shows promise in interacting with metabolic pathways and directly with mitochondria. Preliminary data support the connection of miRNA-133b to mitochondrial protein synthesis possibly through its interactions with the energy sensing heterotrimer AMPK.

The purpose of this investigation is to determine the roles of miRNA-1 and -133b in the dysfunctional protein synthesis and metabolic control of insulin resistant skeletal muscle. While exercise and physical activity can never be replaced in improving health, insulin resistant skeletal muscle seems to respond differently to the stimulus of exercise. Understanding the roles of skeletal muscle miRNAs during insulin resistance will grant us insight into how exercise may act to positively influence health, especially in this disease state.

II. Review of Literature

Since their initial discovery as temporal controllers of developmental stages in Ceanohrabditis elegans (C. elegans) in 1993 (R. C. Lee et al.), the knowledge base surrounding microRNAs (miRNAs) has grown substantially. The research into these important controllers of gene expression still create more questions than are answered. Questions about the mechanisms of generation, coordination and localization of miRNAs bring even more complexity into the realm of inquiry. Skeletal muscle is the most metabolically active tissue in the human body (DeFronzo & Tripathy, 2009), therefore control over metabolic and growth processes by miRNA in this tissue may prove critical to whole body health. A group of miRNAs that are specifically expressed in skeletal muscle (termed myomiRs) have large implications for controlling metabolic activity and health of an entire organism (Guay et al., 2011; Lagos-Quintana et al., 2002). Recent developments in the scientific literature warrant review of certain aspects about their activity. As such the purpose of this review is to outline: the interwoven generation and functions of the muscle specific miRNAs -1, -133, and -206, their specific roles in muscle cell generation, growth, anabolism and metabolic health, and the cellular localization and interactions of these miRNAs to the mitochondria.

Generation and activity of miRNAs

The generation and processing of miRNAs includes multiple steps beginning with the promoting of the *pri*mary transcript (pri-miRNAs), the nuclear processing into a *pre*cursor structure (pre-miRNAs), export to the cytoplasm, Dicer cleavage into a mature miRNA, and development and activity of the *R*NA-*I*nduced *S*ilencing *C*omplex (RISC)(Cullen, 2004; Ha & Kim, 2014). After these steps occur, the mature miRNA generally works in the RISC complex to bind, often imperfectly, a complimentary mRNA to cause down regulation in the content of that

gene (David P. Bartel, 2009). Possible new roles of pre-miRNAs and miRNAs are emerging in mitochondrial gene translation as well (Barrey et al., 2011).

Transcription of miRNAs. miRNA transcription has been linked to RNA polymerase III but is primarily controlled by RNA polymerase II (Borchert, Lanier, & Davidson, 2006; Y. Lee et al., 2004) while the promoting of transcription is less understood. As research surfaces focusing on miRNA promoters, it has helped elucidate the genomic origins of miRNAs. The current understanding is that pri-miRNA transcripts can be classified by having intronic origins from other, already processed messenger RNA (mRNA) transcripts, from within the coding regions of a host gene, or from non-coding intergenic regions (Baskerville & Bartel, 2005; Marsico et al., 2013). Additionally, recent attempts to recognize miRNA promoters (by PROmiRNA analyses) has unveiled that intronic miRNAs can have promoting sequences independent of the host gene disconnecting the expressions of the miRNA from that of its respective host (Marsico et al., 2013). Chromatin condensation, CpG islands, TATA box binding affinity, length of the transcription start site, and distance to pre-miRNA have all been utilized to identify miRNA promoter sites though each origin of miRNAs differ on these aspects (Marsico et al., 2013). Finally, certain transcription factors are known to up and down regulate miRNA transcription (Ha & Kim, 2014). Specifically in skeletal muscle, the myogenic regulatory factors (MRFs) MyoD and myogenin control the transcription of miRNAs -1, -133s, and -206 (Rao, Kumar, Farkhondeh, Baskerville, & Lodish, 2006) making these miRNAs muscle specific (Lagos-Quintana et al., 2002). Reliable and sensitive identification of promoter sequences of pri-miRNA transcripts is a large barrier to identifying transcription factors controlling miRNA genesis (Schanen & Li, 2011).

One of the first characterizations of miRNAs from C. elegans was able to identify mature sequences based on the clustering of multiple miRNAs to one pri-miRNA sequence(Lau, Lim, Weinstein, & Bartel, 2001). Though pri-miRNAs are less understood because of their transience, the transcript length of pri-miRNAs, usually 5-10kb, can be as long as 100kb (Schanen & Li, 2011). This characterization of miRNAs into clusters on DNA and pre-mRNA transcripts is of interest to investigations of muscle specific miRNAs -1, -133a, -133b, and -206. These miRNAs form pairs that originate only a few thousand base pairs away from one another. Based on RNAfold (Hofacker, 2003) predicted secondary structure of these RNA transcripts, the pair miRNA-1 and -133a can generate their pre-miRNA structure in close proximity to one another. This is replicated in the pairing of miR-133b and -206 (Figure 1).

Nuclear and cytoplasmic processing. Further processing of pri-miRNAs into the shorter, more detectable pre-miRNA structures is under the control of the Microprocessor complex consisting of the pri-miRNA, the RNase III Drosha, and a cofactor, DGCR8, in the nucleus (Han et al., 2004). This complex consisting of a Drosha homodimer binding to DGCR8 is able to consistently identify the pri-miRNA stem-loop structures and separate the pre-miRNA from them (Gregory et al., 2004). Recently, Auyeung et al. (2013) demonstrated that a CNNC motif ~17bp downstream of the Drosha cleavage site is highly conserved in mammals and enhances processing. This pre-miRNA is then enveloped by Exportin5 and uses a Ran-GTP gradient to transport the pre-miRNA across the nuclear envelope into the cytoplasm, releasing the unbound pre-miRNA and dissociating the Ran and hydrolyzed GDP (Bohnsack, Czaplinski, & Gorlich, 2004). Cytoplasmic pre-miRNA processing is facilitated by the ~200kDa RNase III endonuclease Dicer (Ketting et al., 2001). The structure of Dicer allows it to function as a molecular ruler by binding the dsRNA with ~25bp (65Å) between the PAZ domain (binds the

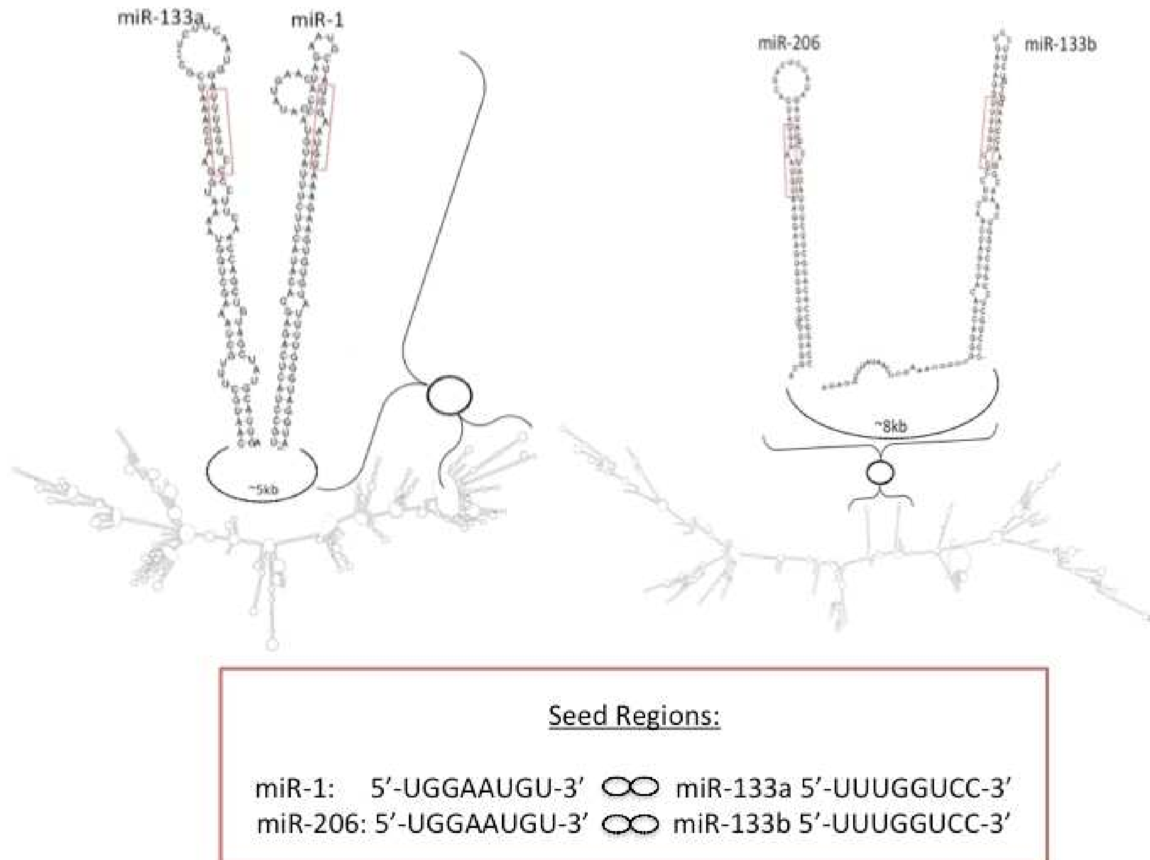

Figure 1. Theoretical RNAfold predicted secondary structures of two separate, highly-conserved pri-microRNA sequences. These sequences show the close proximity of miR-1 to miR-133a as well as miR-206 to miR-133b and the redundant seed sequences of each pair. Seed sequences are outlined.

two pre-miRNA termini) and the RNase III domain which cleaves the hairpin loop (Macrae et al., 2006) resulting in a duplex of two ~22bp ssRNA sequences (Vermeulen et al., 2005).

RISC complex. The formation of the RISC complex is thought to be under the primary control of Argonaute-2 (AGO2) in humans (Forstemann, Horwich, Wee, Tomari, & Zamore, 2007; Meister et al., 2004). This specificity seems to be due to the incomplete complementarity of miRNAs in contrast to other siRNAs; this inhibits the slicing activity of the duplex (Matranga, Tomari, Shin, Bartel, & Zamore, 2005). A predominant mode of RISC complex formation is the

‘Dicer-dependent AGO loading’ model in which the miRNA duplex, still bound to Dicer, associates with AGO to form a pre-RISC complex (X. Liu, Jin, McManus, & Mourelatos, 2012). There is a Dicer-independent AGO loading pathway involving a miRNA Precursor Deposit Complex (miPDC)(X. Liu et al., 2012). Based on thermodynamic stability, AGO loading of the mature miRNA guide strand on the RISC is favored over the miRNA passenger strand (Schwarz et al., 2003; Steiner et al., 2007). The duplex is actively transferred to the AGO protein where passive unwinding, without passenger strand cleavage, takes place (Kawamata, Seitz, & Tomari, 2009; Maniataki & Mourelatos, 2005; Yoda et al., 2010). The result is a mature RISC complex with AGO protein bound to a single stranded, mature miRNA.

Target recognition and repression. A consensus ‘seed-region’ exists in miRNAs where perfect Watson-Crick complementarity exists between the 5’ bases 2-8 (Doench & Sharp, 2004; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003; Watson & Crick, 1953). The mRNA targets of miRNAs are mainly determined based on this heptamer complementarity, an allowance for G-U binding, a high Z score of the folding free energy *G*, and conservation across organisms (Betel, Wilson, Gabow, Marks, & Sander, 2008; Lewis et al., 2003). Binding of 2-8bp of mRNA to the 5’ seed region is sufficient because this short sequence is prearranged in an α-helix open for binding where more bases could result in suboptimal geometric orientation (D. P. Bartel, 2004; Mallory et al., 2004). Where extensive complementarity exists, the AGO protein will lose affinity for the miRNA and allow additional helical twists around the target (David P. Bartel, 2009). Furthermore, pairing of the seed-region at residues 13-16 could be favorable by allowing additional binding sites to the mRNA without requiring the energy loss by release of the AGO binding and conformational twisting of mRNA (David P. Bartel, 2009). Once bound, the RISC can degrade an mRNA by deadenylation of the poly(A) tail in an AU-Rich Element (ARE)

dependent manner (usually seen in perfectly matching sequences) (van den Berg, Mols, & Han, 2008), or more commonly, the RISC complex can inhibit translation initiation by targeting the eIF4F Cap-Binding Complex (Mathonnet et al., 2007; Pillai et al., 2005).

miRNA-1

miRNA-1 is highly conserved and specifically expressed in striated muscle fibers (Lagos-Quintana et al., 2002). miRNA-1 appears to play several significant roles in these tissues. Of note, the importance of miRNAs in temporal development was proven during their discovery by addressing lin-4 anti-sense regulation of lin-14 during developmental stages in C. elegans (R. C. Lee et al., 1993). miRNA-1 similarly contributes significantly to myoblast differentiation into myotubes (Chen et al., 2006; Nakasa et al., 2010; Zhang et al., 2014). Not only does miRNA-1 expression increase dramatically during differentiation but exogenous overexpression of miRNA-1 expedites the process as well (Chen et al., 2006). Wang et al. (2006) demonstrated that miRNA-1 inhibits HDAC4 activity by decreasing the amount of its mRNA translated, effectively upregulating myotube differentiation. This research into myoblast proliferation and differentiation with miRNAs ties directly into the effects that these controllers have on muscle growth and protein synthesis.

miRNA-1 has also been implicated in controlling striated muscle hypertrophy, though to-date, this has been restricted to cardiac myocytes and pathological hypertrophy. Sayad et al. (2007) showed that upon transverse aortic constriction (TAC) induced cardiac hypertrophy, miRNA-1 was diminished for up to 14 days. Their cell culture experiments showed atrial natriuretic factor (a marker of cardiac hypertrophy) is diminished with transient miRNA-1 overexpression. Similarly, Elia et al. (2009) described reduced miRNA-1 content by ~50% during TAC and Akt overexpression models of cardiac hypertrophy. They further used a reporter

assays in multiple experiments of mutant miRNA-1 and IGF-1 gene transfection to demonstrate inhibition of miRNA-1 on IGF-1. They show a negative correlation between miRNA-1 levels to left ventricular mass index and maximal wall thickness in both acromegaly patients and healthy donors. To date, this study has proven paramount in the modulation of protein synthesis by miRNA-1 via inhibition of IGF-1 though no direct measures of protein synthesis were obtained. miRNA-1 appears important in muscular damage and regeneration. Nakasa and colleagues (2010) demonstrated that miRNAs -1, -133, and -206 are decreased by ~75% upon laceration of the tibialis anterior and gradually recover over 7 days. Building on evidence of enhanced proliferation in C2C12 myoblasts, they used a muscle specific miRNA overexpression cocktail (miRNAs-1,-133, and -206) injection in hopes of accelerating muscle cell regeneration post-injury *in-vivo*. They successfully showed an increase in myogenic markers and increased morphological recovery with a decreased amount of fibrosis. However, it should be noted that the degree of miRNA overexpression was undefined as well as the contributions of the individual miRNAs on regeneration, thereby leaving some questions unanswered.

McCarthy and Esser (2007) showed increases in pri-miRNA transcripts of miRNA-1 following seven days of functional overload induced hypertrophy although the mature sequences were decreased. They conclude that the processing of miRNAs in skeletal muscle is interrupted during growth in order to reduce translational repression thereby increasing protein synthesis. Another instance of dysregulated pri-miRNA processing is shown by Drummond et al. (2008) in humans where aged men had increased levels of pri-miRNA-1 but no change in the mature transcript. Additionally, they showed that in response to an acute bout of resistance exercise, miRNA-1 was decreased, purported to be promoting an anabolic stimulus.

These investigations clearly demonstrate the importance of miRNA-1 in muscle cell proliferation and differentiation along with regeneration from injury. This has implications for injuries associated with normal exercise bouts to traumatic muscle injuries such as are seen in military combat scenarios. Additionally, the direct effect of miRNA-1 on IGF-1 signaling (Figure 2) as shown in cardiac muscle could make a profound impact on muscle growth and atrophy as seen from resistance training induced hypertrophy to cancer cachexia and spaceflight induced atrophy.

miRNA-206. miRNA-206 and miRNA-1 contain complementary, highly conserved, identical 5' seed region sequences. Based on TargetScan (Friedman, Farh, Burge, & Bartel, 2009), PicTar (Lall et al., 2006), Miranda (Betel et al., 2008), and miRBase (Griffiths-Jones, Saini, van Dongen, & Enright, 2008) target predictions, stringent seed pairing is the most important factor used to determine mRNA targeting. This results in concomitant predictions of miRNA-206 and miRNA-1 binding to complimentary mRNA targets (Figure 2). miRNA-206 has appeared in the literature less frequently than miRNA-1 to date though it is worth mentioning a few key findings. miRNA-206 has been shown to follow the same expression patterns of miRNA-1 during C2C12 differentiation (Chen et al., 2006). Kim et al. (2006) provided concrete evidence that miRNA-206 promotes differentiation and is necessary for proper formation of myoblasts by directly inhibiting DNA polymerase α. Like, miRNA-1, miRNA-206 is enhanced (though to a much greater extent) during hypertrophy (McCarthy & Esser, 2007). Olson and colleagues (2012) observe the effects of miRNA-206-KO in exacerbating muscular dystrophy elucidating the pathophysiology of the disorder. Finally, miRNA-206 has yet to be detected in or around the mitochondria, but it may likely play a role in oxidative metabolism as its expression is greatly

increased in the oxidative soleus muscle of rats compared to mixed fiber plantaris. (McCarthy & Esser, 2007); the specific mechanisms responsible for this are yet to be elucidated.

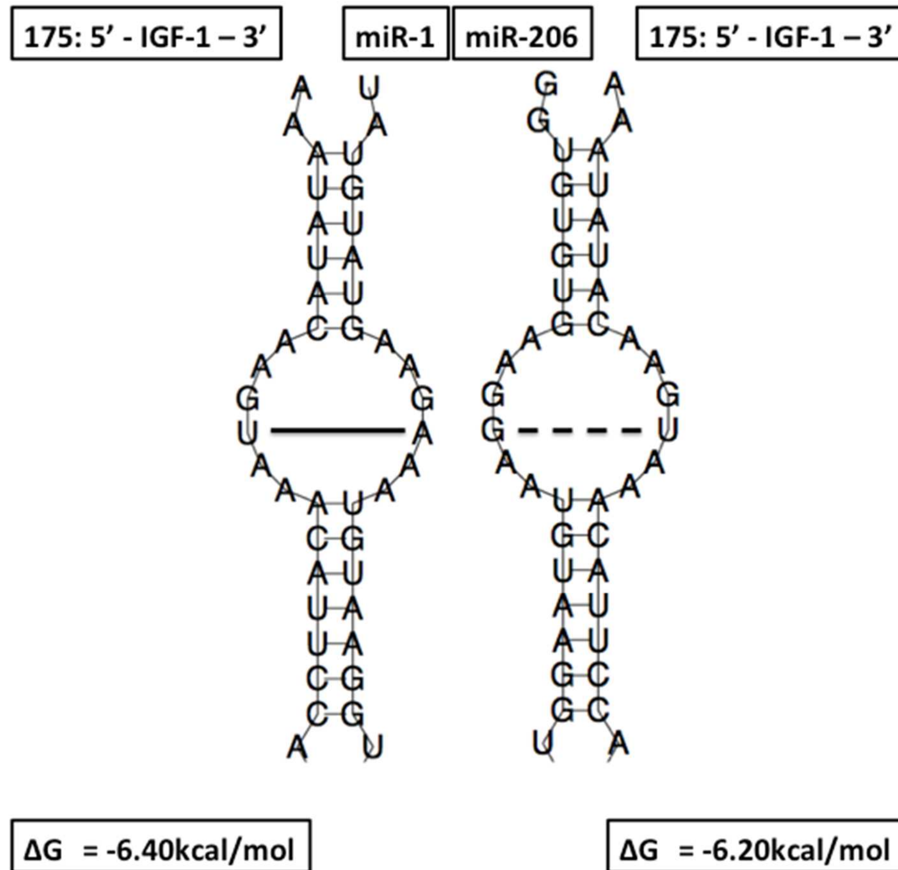

Figure 2. miR-1 and miR-206 show near identical binding patterns to the coding sequence of IGF-1. This IGF-1 sequence and the miRNA seed sequences are conserved from rodents to humans. Free energy predictions are based on physiological temperature and pH.

miR-133

The miRNA-133 group is composed of miRNAs-133a and -133b, which have homologous mature miRNA sequences save for a single 3' (non-stem sequence) A-G (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006; Griffiths-Jones et al., 2008). As muscle specific miRNAs, these genes have been implicated in many similar scenarios as the previously mentioned myomiRs, though some evidence suggests a far different role. While similar patterns

as miRNA-1 have emerged with miRNAs-133 during myoblast differentiation (Nakasa et al., 2010) and *in-vivo* muscle hypertrophy (McCarthy & Esser, 2007), some important distinctions have been shown. While miRNA-1 had a more direct involvement in myoblast differentiation, miRNA-133 directly inhibits serum response factor (SRF) leading to a state favoring proliferation (Chen et al., 2006). While miRNA-1 may be linked to muscle regeneration and growth, miRNA-133 has been widely implicated in metabolic function.

Like other important oxidative controllers such as PGC-1α, often times, co-expression can be seen in muscle tissue as well as brown adipose tissue and this is the case for most of the myomiRs (Puigserver et al., 1998; Trajkovski, Ahmed, Esau, & Stoffel, 2012). Importantly, Trajkovski and colleagues (2012) showed miRNA-133 targets PR domain containing 16 (Prdm16) in brown adipose tissue and subsequently affects levels of the metabolic proteins Pgc1α, PPARγ, and UCP-1, as well as oxygen consumption (W. Liu et al., 2013; W. Liu & Kuang, 2013). This connection to lipid metabolism has not yet been replicated in skeletal muscle. In cardiomyocytes, miRNA-133 plays a role in cardiac hypertrophy of streptozotocin induced diabetic rats further connecting it with genetic control in metabolic diseases (Feng et al., 2010). In human skeletal muscle myoblasts, miRNAs-133a and -133b were significantly detected in highly purified mitochondria (Barrey et al., 2011) further bringing out this connection to metabolic activity in skeletal muscle. While this evidence exists, it should not downplay the importance of miRNA-133 in muscle growth, which has been shown in cardiac muscle through effects on RhoA, Cdc42, and Nelf-A/WHSC2 (Care et al., 2007) and skeletal muscle regeneration (Nakasa et al., 2010).

Mitochondria-targeted miRNAs.

Due to the efficient transcription and translation of prokaryotic genes, the lack of 5’ caps and 3’ poly(A) tails, and absence of introns, miRNAs seem to be specific to eukaryotic cells. This reasoning leads one to believe that miRNAs in muscle cells are specific to nuclear genome transcripts and absent from the mitochondrial DNA based on the similarities between mitochondrial and prokaryotic gene processing. However, recent investigations have found that pre-miRNAs, mature miRNAs, and AGO proteins are colocalized to the perimeter of the mitochondria (Bandiera, Mategot, Girard, Demongeot, & Henrion-Caude, 2013). Furthermore, in highly purified mitochondria and mitoplasts, pre-miRNAs and miRNAs are detected and effect the mitochondrial genome (Barrey et al., 2011; Towheed, Markantone, Crain, Celotto, & Palladino, 2014). As many as ~160 miRNAs were significantly detected in skeletal muscle mitochondria when high amounts of input were used (Barrey et al., 2011). Zhang et al. (2014) used various approaches to delineate a role of miRNA-1 in the mitochondria during muscle cell differentiation. Their group and others have noted a drastic increase in mature miRNA-1 content upon myoblast differentiation (Chen et al., 2006). Interestingly, miRNA-1 and AGO2 were detected inside of the mitochondria after differentiation. The ratio of mitochondrial miRNA-1 to cytosolic miRNA-1 seemed to increase indicating a shift of miRNA-1 (and potentially others) into the mitochondria. GW182 is a protein that interacts with AGO2 in the cytoplasm but is not found in mitochondria; its association is required for deadenylation of an mRNA, and its dissociation from AGO2 is related to activation of translation (Iwasaki & Tomari, 2009; Vasudevan, 2012). Based on the lack of: GW182 in mitochondria, 5’ methylated caps, and 3’ poly(A) tails in mtDNA transcripts (not dissimilar to prokaryotes), the mitochondrial RISC (mtRISC) appears to enhance translation contrary to that of the cytosolic RISC (Iwasaki &

Tomari, 2009; Smits, Smeitink, & van den Heuvel, 2010; Vasudevan, 2012). This shift of miRNAs into the mitochondria during a temporal event such as differentiation regulates a simultaneous enhancement of cytosolic and mitochondrial mRNA translation (Zhang et al., 2014).

Indirectly, multiple miRNAs have been shown to work on nuclear transcribed mRNAs to affect the mitochondria. Aoi et al. (2010) demonstrated the direct effects of miRNA-696 in mouse skeletal muscle to promote mitochondrial biogenesis through *Pgc-1α* thereby enhancing aerobic exercise capacity. miRNA-181 was co-localized to the mitochondrial membrane (Barrey et al., 2011) and negatively affects multiple mitochondria-encoded genes of the cyctochrome oxidase family, downstream oxygen consumption and ROS generation (Das et al., 2014). Much attention has also been given to miRNA-23a because of its strong predicted binding of *Pgc-1α* though Wada and colleagues (2014) provided strong evidence against this assertion by only showing a shift in muscle fiber type with no concomitant enhancement of exercise capacity or response to aerobic training in miRNA-23 overexpressing mice. These insights into miRNA control over mitochondria directly and indirectly and leave much to be done to clarify how miRNAs might control metabolic health.

Conclusions and Future Direction

It is clear that these small non-coding modulators of gene activity control multiple aspects of skeletal muscle biology (Figure 3). Each of these muscle specific miRNAs: miRNA-1, -133a, -133b, and -206, play important roles in skeletal muscle proliferation and differentiation through various myogenic promoters. Phenotypically, evidence exists for miRNA modulation of skeletal muscle regeneration, hypertrophy/atrophy, and mitochondrial activity. Still many questions remain. Do these myomiRs modulate gene expression enough to affect whole organism

physiology? What role do these miRNAs play during metabolic deficiency as seen in type II diabetes? Is it certain that mitochondrial DNA does not produce miRNAs? Aside from miRNA-1, what other myomiRs directly control mitochondrial mRNA translation? What controllers locate miRNAs to the mitochondrion and how are they transported through the mitochondrial membranes? What is the significance of the anabolic shift of miRNAs to the mitochondria? Importantly, what possibilities exist for translational applications of miRNAs as therapeutic targets of disease as has been implicated (Carey & Kingwell, 2009; Tseng, Cypess, & Kahn, 2010) and what application do miRNAs have in controlling muscle atrophy, hypertrophy, and insulin sensitivity as has been questioned by others (Goljanek-Whysall, Sweetman, & Munsterberg, 2012; Guller & Russell, 2010; Kirby & McCarthy, 2013; Nielsen et al., 2010; Russell et al., 2013; Zacharewicz, Lamon, & Russell, 2013)?

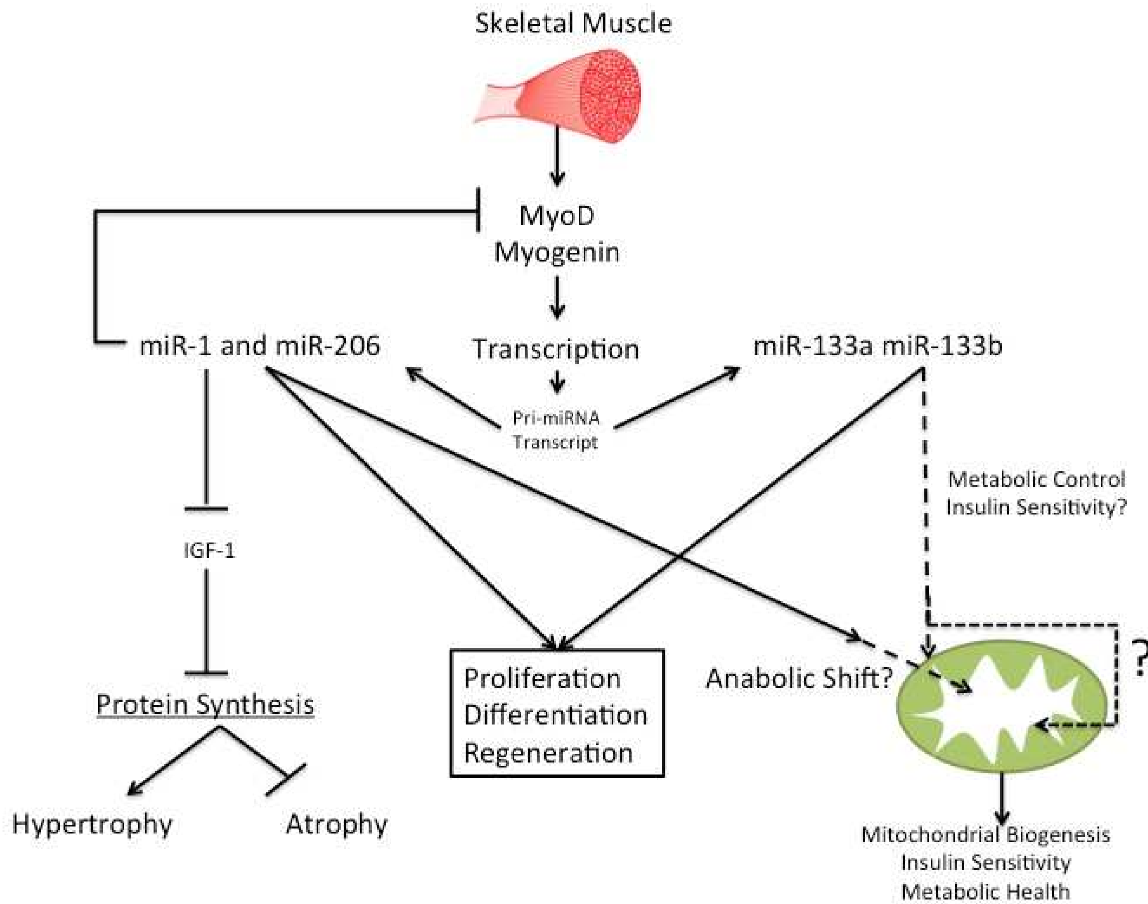

*Figure 3***.** Control of muscle specific microRNAs by myoD and myogenin could lead to coordination of expression between miRNA-1, -133, and -206. These microRNAs play important roles in proliferation, differentiation, regeneration, protein synthesis and mitochondrial network health.

III. Methods

C2C12 cell culture

C2C12 mouse skeletal muscle myoblasts were used throughout cell culture experiments. Cells were plated in 6-well cell culture plates at 50,000 cells per well in growth medium (79% DMEM, 20% FBS, 1% P/S) and were transfected 24 hours later. Transfections were done using Lipofectamin2000 reagent with DNA plasmid in a 3:1 ratio (v/wt) according to manufacturer protocols. Lipofectamine/plasmid complexes were dissolved in serum-free Opti-MEM and added to cells. After 5 hours of incubation, media was replaced with growth medium. Cells grew until 90% confluent and harvested for protein and RNA or switched to differentiation medium (92% DMEM, 2% HS, 1% P/S, 5% HEPES, 0.25% insulin, 0.25% transferrin). Cells were allowed to differentiate for 5 days and then harvested for protein and mRNA. Immediately prior to harvest, media were removed, cells rinsed with PBS, and 300nM DAPI in PBS was added to cell for 5 minutes to stain nuclei.

DNA-plasmids

Plasmids were purchased through OriGene (Rockland, MD) and bacteria were transformed to express plasmid DNA complimentary to an empty vector control (pCMV-miR), miR-1 (pCMV-miR-1), and miR-133b (pCMV-miR-133b) under a CMV promoter. DNA plasmids were amplified in DH5α competent bacterial cells and isolated using commercially available kits (Invitrogen, Life Technologies). Each plasmid co-expresses a GFP reporter protein that will be used to assess transfection efficiency and visualize cells.

Non-isotopic measurement of protein synthesis

To measure relative rates of protein synthesis, the SUnSET technique was utilized as described previously (Goodman & Hornberger, 2013; Goodman et al., 2011). Briefly, cells were

treated with 1uM puromycin dihydrochloride (540222, EMD Millipore, Darmstadt, Germany) and incubated for 30 minutes. After 30 minutes cells were harvested for protein, whole cell protein will be transferred to PVDF membranes (see protocols below), probed with primary mouse IgG MAb-2a anti-puromycin (1:20,000)(MABE343, EMD Millipore, Darmstadt, Germany) overnight at 4°C followed by secondary incubation in goat anti-mouse IgG MAb-2a specific HRP conjugated antibody for 1 hour at room temperature (1:20,000; 15-035-206, Jackson ImmunoResearch, West Grove, PA). After 30 minutes of washing in TBS-T, membranes were imaged on Fluorochem Western Blot Imager. The normalized optical density for the entire lane of each sample will be used to calculate rates of protein synthesis relative to control groups and normalized to Ponceau S stain.

Gene expression analysis

RNA was extracted from cells by scraping in 1mL of Tri reagent and isolated using a combination of chloroform-guanidine thiocyanate organic isolation and PureLink gel-silicate membrane columns (Life Technologies, Grand Island, NY) as per manufacturers protocol. RNA concentration was determined using a Take3 plate (Winooski, VT) at 260 and referenced at 280nm. Following DNAse treatment of RNA, cDNA was synthesized using SuperScript VILO (Life Technologies, Grand Island, NY) as per manufacturers protocols using 1ug of total RNA. For mRNA expressions, real time qPCR was utilized using SYBR green dyes and appropriate primer pairs. All Ct values will be normalized to expression of 18S rRNA and quantified and normalized by the ΔΔCt method. For miRNA analysis, reverse transcription using specific miRNA stem-looped primers was performed prior to qPCR analysis of miRNAs using Taqman probes and was normalized to snU6 as an endogenous control.

Western Blot analysis

Using a protein buffer (80mM DTT, 0.57mM 2-ME, 4.5% w/v SDS, 45% w/v glycerol, 0.04% w/v bromophenol blue, 2x protease inhibitor cocktail, 2x phosphatase inhibitor cocktail), cells were scraped, filtered of particulate, and denatured at 95°C. Concentrations were measured using the DC form of the Lowry Assay with triplicates. 40ug of protein will be run on 10% SDS-PAGE, transferred to PVDF membranes, and blocked in 5% BSA for 60 minutes. Primary anti-bodies for puromycin, p70S6K, p-p70S6k$_{Thr\text{-}389}$, Akt, p-Akt$_{Ser\text{-}473}$, COXIV, AMPK, and p-AMPK$_{Thr\text{-}172}$ will be diluted in 3% BSA dissolved in TBS-T and membranes incubated overnight. Secondary HRP conjugated anti-bodies were diluted in 3% BSA in TBS-T for 60 minutes and membranes were imaged using WesternSURE premium ECL on a Fluorochem blot scanner. Protein expressions were normalized to Ponceau S stain.

Florescent microscopy

Cells were imaged using a Nikon Eclipse Ti-S inverted fluorescence microscope and analyzed on Nikon Imaging Software (Nikon, Melville, New York). Multiple images were taken to cover the entire well. Images for DAPI were used to determine proliferation and FITC (GFP label on miR plasmid) were used for myotube diameter. An overlay of DAPI and FITC will be used for analysis of myotube fusion or multinuclieation of cells.

Statistical analyses

A 2x2 ANOVA was tested to compare empty vector, miR overexpressed pre-differentiation and post-differentiation using SAS. The chance of type I error, α, was set at $p \leq 0.05$. Dependent variables of interest are myotube diameter, puromycin incorporation, IGF-1 expression, Akt phosphorylation, p70S6K phosphorylation, AMPK phosphorylation, COXIV content. The independent variables were plasmid group (empty vector, miR-1, and miR-133b) coupled with no treatment control.

IV. Results

C2C12 myoblasts were successfully transfected using lipofectamine-mediated gene transfer of miR-1 (~23-fold higher than control; $p < 0.001$; Figure 1A) and miR-133b (~6-fold higher than control; $p = 0.021$; Figure 1B).

miR-1 Overexpression Alters Protein Synthetic Signaling

Basal puromycin incorporation into the polysome was unaltered with miR-1 overexpression ($p > 0.05$; Figure 2A). Greater miR-1 did not change the content of *Igf-1* mRNA ($p = 0.2865$, Figure 2B). ~ 25% Less phosphorylation of Akt was seen ($p = 0.052$, Figure 2C) in miR-1 compared to empty vector pCMV-miR (control). No differences were seen in DEPTOR protein content between miR-1 overexpression and scrambled miR control (Figure 2D). Further downstream, total p70S6K1 content was 74% of that measured in the control ($p < 0.001$; Figure 2E). The ratio of phosphorylated p70S6K1: Total was also ~55% less ($p = 0.013$, Figure 2F) compared to the control. Total 4E-BP1 protein content was ~3-fold higher ($p = 0.017$, Figure 2G) in miR-1 overexpression compared to the control; however, the ratio of phosphorylated to total 4E-BP1 was unchanged ($p = 0.85$, Figure 2H).

miR-133b Overexpression Does Not Alter Protein Synthesis

Puromycin incorporation into the polysome was unaltered with miR-133b overexpression ($p > 0.05$; Figure 2). miR-133b overexpression did not change the content of *Igf-1* mRNA ($p = 0.2243$; Figure 2B), phosphorylation of Akt ($p = 0.319$; Figure 2C), nor DEPTOR protein content ($p = 0.474$, Figure 2D). Further downstream, total p70S6K1 content was 50% ($p < 0.001$; Figure 2E) of that measured in the control. The ratio of phosphorylated p70S6K1 approached significance with ~70% ($p = 0.076$; Figure 2F) of that of the control.

Overexpression of miR-133b had no affect on the total content of 4E-BP1 nor the ratio of phosphorylated to total 4E-BP1 ($p > 0.05$; Figure 2G,H).

miR-1 and 133b Overexpression Alters Mitochondrial Content and Turnover

COX-IV gene and protein content were both ~100% ($p < 0.001$; Figure 4A) and ~130% ($p = 0.0414$; Figure 4A) higher, respectively, in miR-1 overexpression. No significant differences were seen in the protein expression of TFAM or PGC-1α ($p > 0.05$; Figure 4B). COX-IV gene and protein content were both ~45% ($p = 0.0163$; Figure 4D) and ~59% ($p = 0.009$; Figure 4D) higher, respectively, in miR-133b overexpression compared to control. No significant differences were seen in the protein expression of TFAM or PGC-1α ($p > 0.05$; Figure 4E).

pMitoTimer fluorescence for both miRs-1 and 133b shifted towards green with greater overall green fluorescent intensity (~25% and ~35%, respectively; $p < 0.01$; Figure 5A), less red fluorescence intensity (~20% for both; $p < 0.01$, Figure 5B), and a higher green: red ratio (~45% and ~65%, respectively; $p < 0.01$; Figure 5C) when compared to control cells.

V. Discussion

Here, the effects on protein synthesis by overexpressed miRs -1 and -133 are presented. Because of the unclear actions that miRs have directly on mitochondrial content, the amount of mitochondria (as measured by the COX-IV oxidative protein) and related controllers were assessed. IGF-1 protein has already been shown to be downregulated by miR-1, and miR-1 levels have been negatively correlated with muscle size (Elia et al., 2009). The explicit downstream effect of this inhibition is less understood, specifically, the direct affect on rates of total protein synthesis. Aside from protein metabolism, miRs are now being reported as direct enhancers of mitochondrial translation. Individual overexpression of miRs -1 and -133 significantly altered total content and phosphorylation of protein synthetic machinery. These changes, however, did not result in any concomitant change in basal protein synthesis as measured by puromycin incorporation. Additionally, gene and protein contents of COX-IV, a surrogate measure of mitochondrial content, were increased by both miRs and were separate from TFAM or PGC-1α. Together, these results indicate a clear advancement in the knowledge of the effects miRs -1 and -133 have on protein synthesis and on the mitochondrial network. This study has taken clear steps towards understanding the effects of miRs -1 and -133b on protein synthesis and the mitochondrial network through assessment of mitochondrial content and turnover.

microRNA-1 alters protein synthetic machinery but not basal protein synthesis

Though previously not seen at the level of mRNA, miR-1 inhibition has been shown to down regulate IGF-1 protein content (Elia et al., 2009). Downregulated IGF-1 activity should decrease the phosphorylation of Akt leading to a wide range of outcomes, most notably, reduced protein synthesis via mTOR signaling (Rommel et al., 2001). miR-1 suppressed protein synthetic signaling, likely through this interaction with IGF-1 as evidenced by reduced phosphorylation of

Akt and p70S6K1. Additionally, miR-1 overexpression appeared to directly suppress protein synthetic signaling as evidenced by increased total 4E-BP1 and decreased total p70S6K1. These basal modulations in canonical controllers of protein synthesis are not reflected by any differences in the basal rate of mRNA translation, evidenced by lack of difference in puromycin incorporation. The lack in suppression of basal protein synthesis does not exclude miR-1 from being a regulator of stimulated protein synthesis such as seen by insulin, IGF-1 or other hypertrophic stimuli. Further work might determine if contraction-induced or insulin-induced activation of protein synthesis through this pathway is decreased by the lack of machinery available.

p70S6K1 is downregulated by miR-133b

No changes in Insulin/IGF-1 - Akt signaling were seen with overexpression of miR-133b aside from p70S6K1, which showed decreased total expression and phosphorylation. Though it has not been directly tested experimentally, specific transcripts of the RPS6K family contain conserved binding sites of miR-133 (Betel et al., 2008). These changes, like miR-1, did not affect the rate of protein synthesis in the basal state. These combined evidences suggest that while miRs -1 and -133b may impact protein synthetic signaling they do not specifically alter basal protein synthesis.

microRNA-1 and -133b alter the Mitochondrial Network

Mitochondrial content assessed by the COX-IV gene and protein expression was increased in C2C12 myocytes overexpressing either miR-1 or miR-133b. This increase in the amount of mitochondria could promote an oxidative phenotype in the cell similar to that seen in other models such as with the overexpression of PGC-1α (Geng et al., 2010). Though not a direct measurement, the fluorescent shift towards green fluorescence of pMitoTimer following

overexpression of miRs -1 and -133b is indicative of more new mitochondria, and fewer old or oxidized mitochondria and thereby enhanced total quality of the mitochondrial network (Laker et al., 2014). This type of shift has been shown to be indicative of mitochondrial turnover and mitophagy (Hernandez et al., 2013). The primary regulators for mitochondrial biogenesis, PGC-1α, and mtDNA replication, TFAM, were unaltered in both models of overexpression. Therefore, at this time, the mechanisms by which mitochondrial content and quality are regulated by miRs -1 and -133b are unknown.

Mitochondrial translation enhancement directly by miR-1 has been recently reported (Zhang et al., 2014). This has brought attention to how miRNAs may enhance metabolic health of the cell by affecting the mitochondria. Some evidence exists on how miR-1 may enhance mitochondrial complex enzymatic activity of myoblasts without any alteration in nuclear encoded mitochondrial proteins (i.e. COXIV; (Zhang et al., 2014). Retrograde signaling of mitochondrial translation might result in enhancement of mitochondrial content though this has traditionally been connected to cell proliferation (Battersby & Richter, 2013). The detailed interactions between these miRNAs and the mitochondria deserves further investigation, specifically, how miRNA interactions with mitochondrial translation affect mitochondrial content, what mechanisms govern the transport of the miRNA-AGO2 complex through the mitochondrial membranes and aside from miR-1, what other miRNAs undergo similar interactions is yet to be experimentally validated.

Future Directions

This investigation is limited in its application to C2C12 myoblasts and these results may be different if tested in mature, differentiated myotubes *in-vitro* or *in-vivo* with adult muscle fibers. Further assessment of the mitochondrial network could be conducted by assessing

mtDNA content and activity of miRs on mitochondrial mRNA translation should be assessed by testing miR-1 and miR-133b targets of the mitochondrial genome (i.e. Cox1 or ND1 for miR-1). The localization of miRs within the cell should be assessed by in-situ hybridization using miR-1 specific probes or transfection of a fluorophore-linked mature miR transcript that could bind to AGO2. Because this complex can be detected in the cytoplasm or the mitochondrial matrix (Zhang et al., 2014), and is transported across both mitochondrial membranes, it is important to understand the mechanism of this transport as well as the controls that govern a shift from the cytoplasm to the mitochondria as is seen during C2C12 differentiation (Zhang et al., 2014). The presence of this mitochondrial shift of miRs should be studied in different physiological models of metabolic dysfunction such as diabetes or muscular atrophy.

In summary, the myomiRs miR-1 and -133 are sufficient to increase mitochondrial content as measured by COX-IV and would appear to affect the turnover of the mitochondrial network by creating new mitochondria and removing old, damaged/oxidized mitochondria. Additionally, miR-1 downregulates activity of controllers of protein synthesis but basal protein synthesis is not sensitive to these changes.

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Appendix

Figures of Results

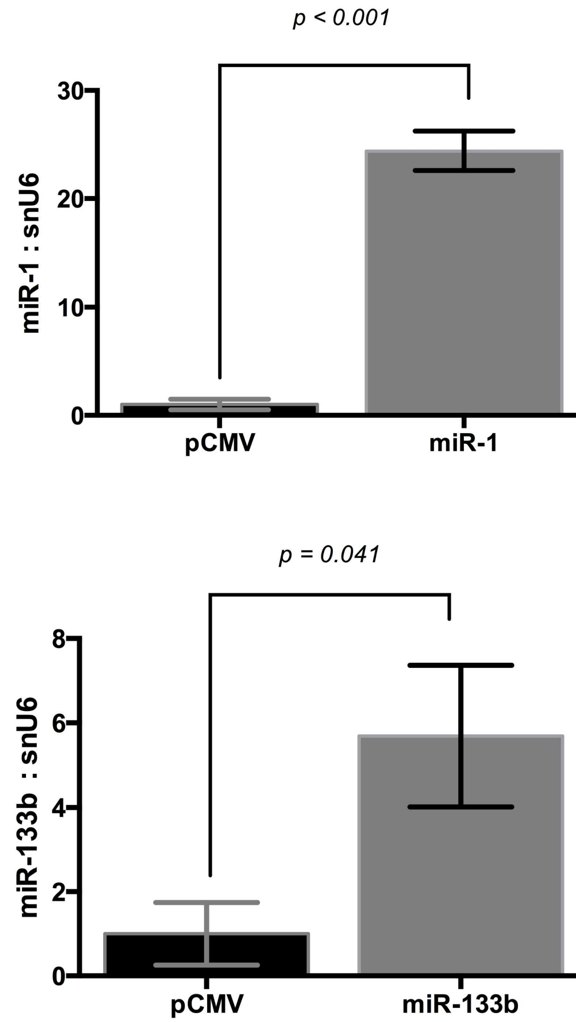

Figure 1. Real-time PCR content of miR-1 and miR-133b for overexpressed miR-1 and miR-133b C2C12 myoblasts, respectively. All values are mean ± SEM.

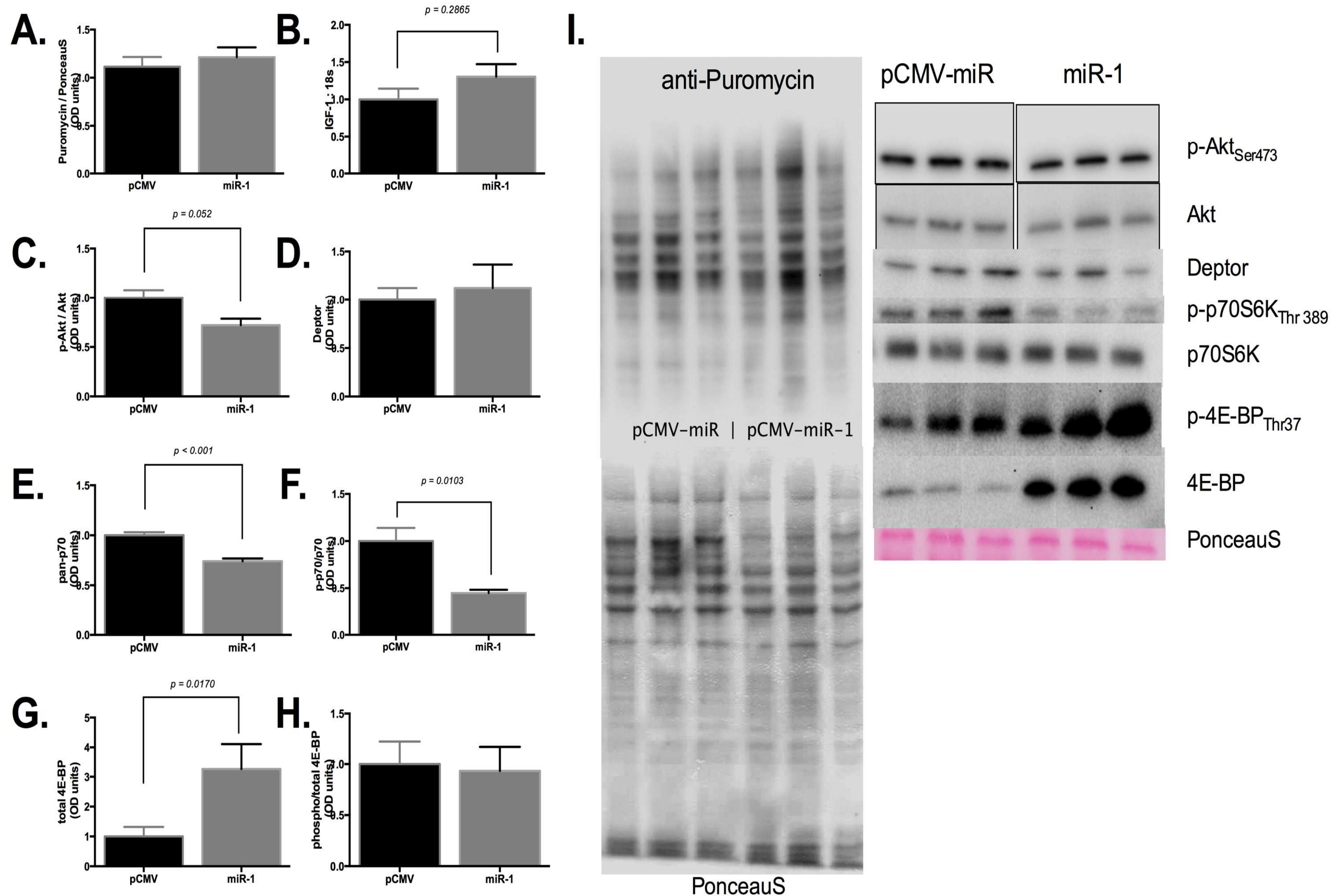

Figure 2. Assessment of protein synthesis by western blot of puromycin as well as protein synthesis signaling cascade in miR-1 overexpressed C2C12 myoblasts and empty vector pCMV-miR transfected controls. A-H) Relative quantification of proteins involved in protein synthetic signaling where all values are mean ± SEM normalized to Ponceau S stain as loading control. I) Representative images. All representative blots are taken from the same image.

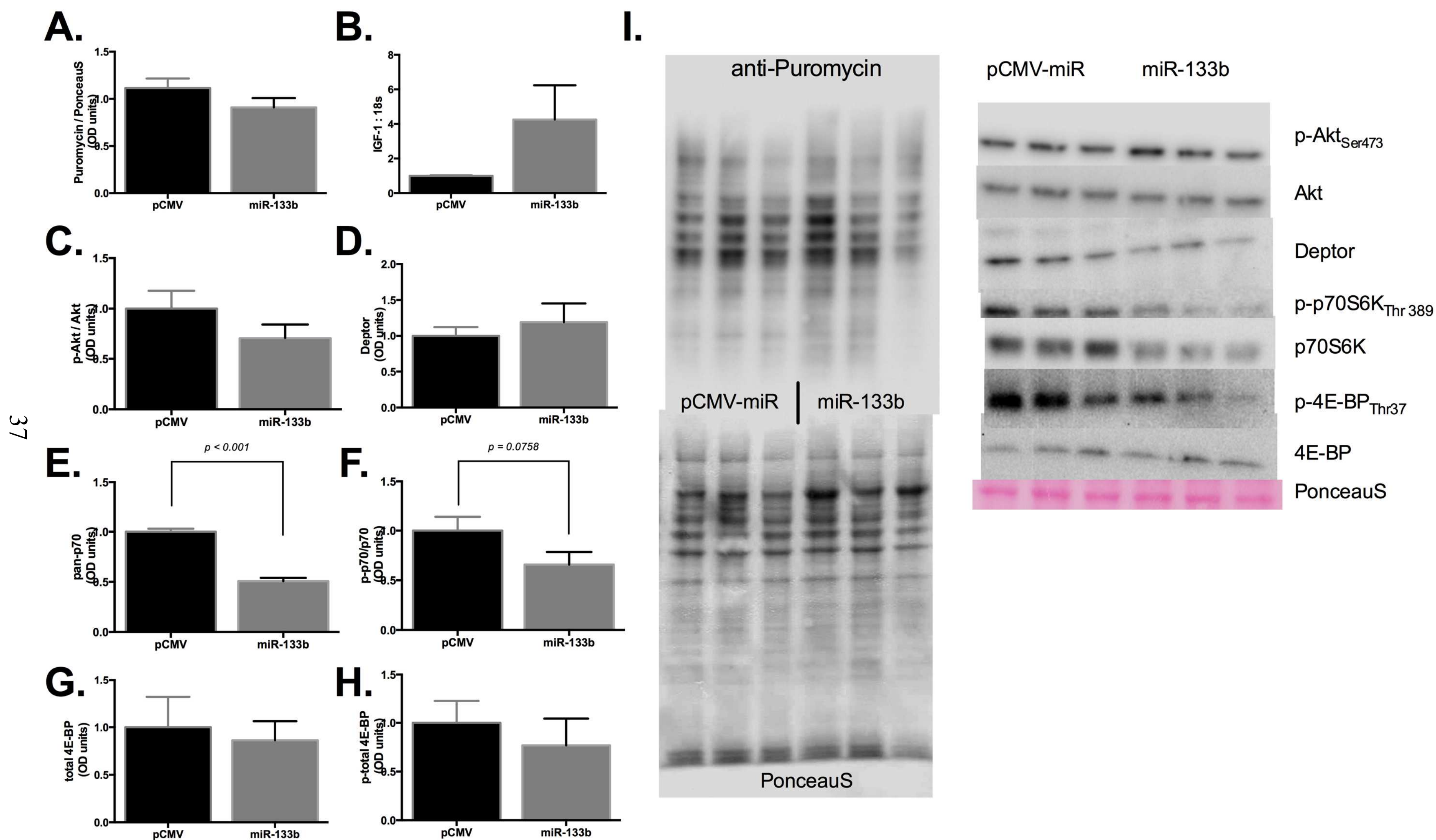

Figure 3. Assessment of protein synthesis by western blot of puromycin as well as protein synthesis signaling cascade in miR-133b overexpressed C2C12 myoblasts and empty vector pCMV-miR transfected controls. A-H) Relative quantification of proteins involved in protein synthetic signaling where all values are mean ± SEM normalized to Ponceau S stain as loading control. I) Representative images. All representative blots are taken from the same image.

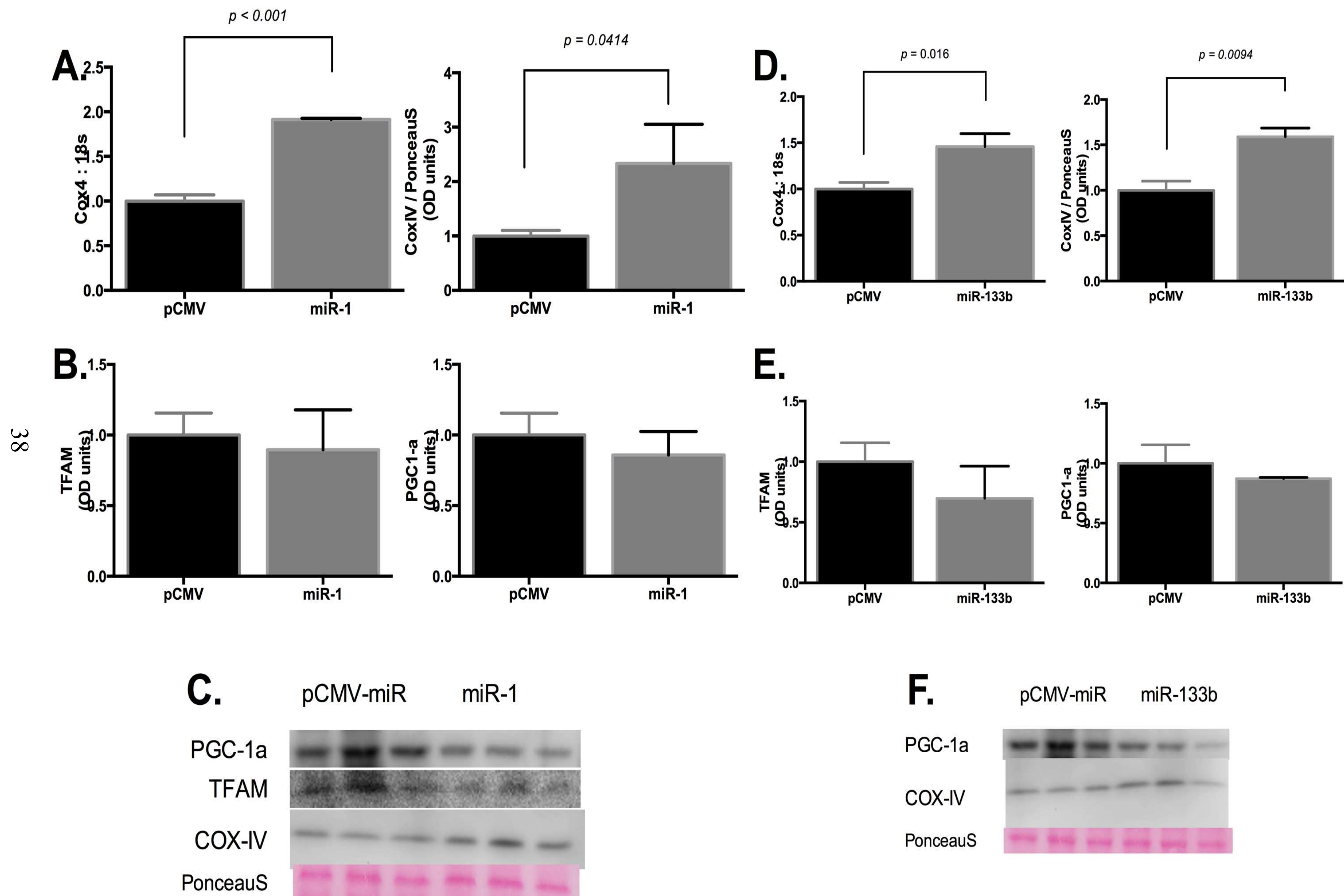

Figure 4. Assessment of mitochondrial content for miR-1 (A) and miR-133b (B) overexpressed myoblasts by Cox4 mRNA and protein, TFAM, and PGC-1α compared to pCMV-miR control. All values are mean ± SEM.

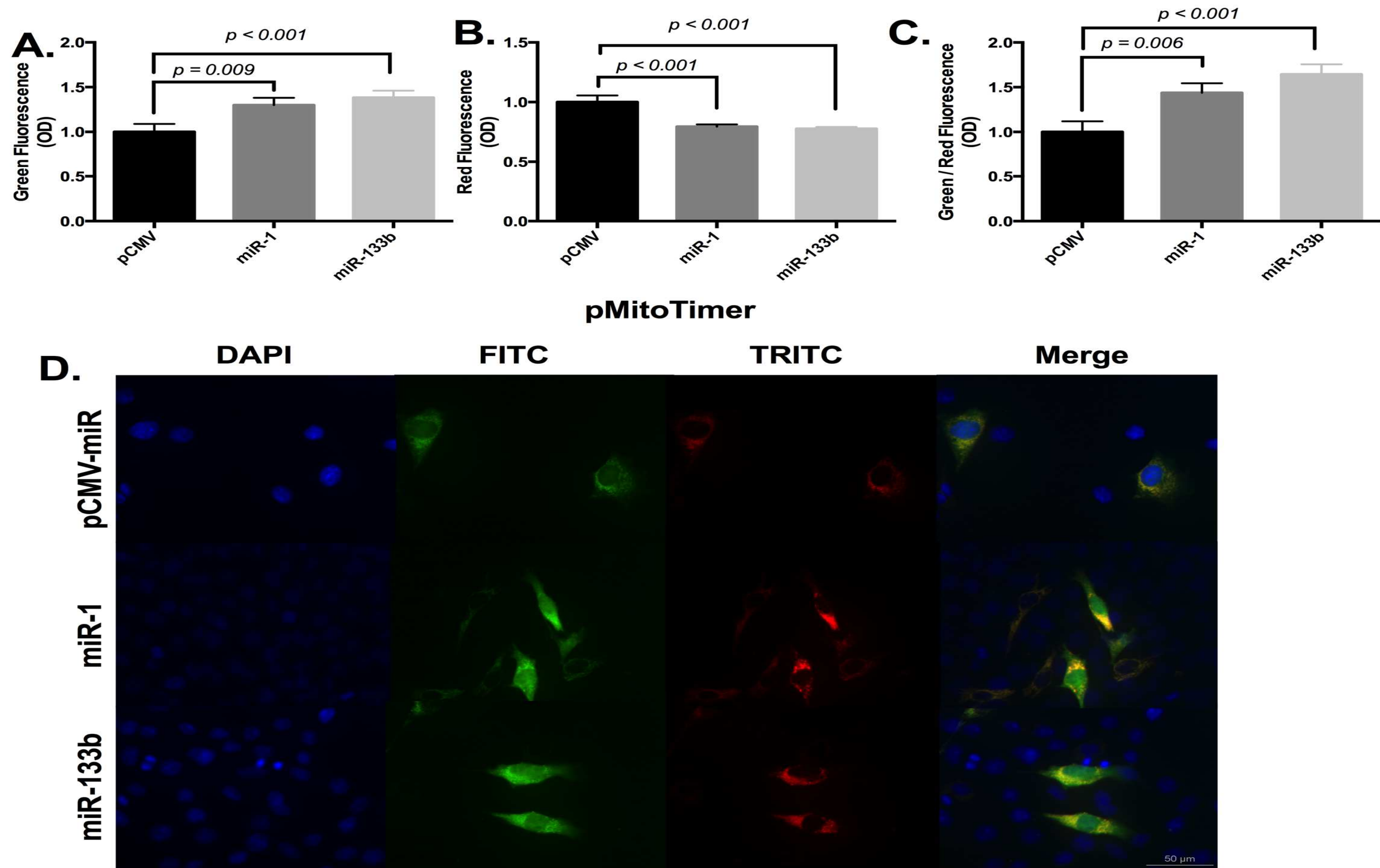

Figure 5. Fluorescent microscopy of C2C12 myoblasts co-transfected with pMitoTimer and either pCMV-miR control, pCMV-miR-1, or pCMV-miR-133b. Quantification of green fluorescence (A), red fluorescence (B), the ratio of green/red (C), and representative images (D). All quantification values are mean ± SEM. Scale bar denotes 50 micrometers.