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MicroRNAs in skeletal muscle biology and exercise adaptation

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Introduction

History

The story of microRNAs (miRNA) begins with the decade long investigation by the Ruvkun laboratory into the regulation of *lin-14* expression during nematode (*C.elegans*) development. In a series of studies that spanned the '90s, Ruvkun and colleagues provided evidence demonstrating that *lin-14* expression was regulated by a post-transcriptional mechanism requiring the interaction between the *lin-14* 3'-UTR and the non-coding RNA *lin-4* [1–6]. In late 2000, Pasquinelli and colleagues reported that another “small temporal RNA” (~ 21 nucleotides) from *C. elegans*, *let-7*, down-regulated *lin-41* expression, and unlike *lin-4*, was detected in a broad range of bilaterian animals including vertebrate, ascidian, hemichordate, mollusc, annelid and arthropod [7]. The finding that *let-7* was phylogenetically conserved was a milestone in the history of the field as it strongly suggested the post-transcriptional regulation of gene expression by small RNAs was more wide-spread and not restricted solely to *C. elegans*. The following year, a series of papers were published describing the identification of 30–50 new miRNAs in the fly, worm and human, providing support for the notion that miRNAs have a prevalent role in the regulation of gene expression in animals [8–11]. As confirmation of this early prediction, the latest release of miRBase (version 19, Aug. 2012; www.mirbase.org) contains 25,141 mature miRNA sequences from 193 species with 2041 and 1281 human and mouse miRNAs, respectively.

Biogenesis—miRNAs are initially transcribed as a primary transcript (pri-miRNA) with the characteristic 5'm⁷G cap structure and 3' poly(A) tail of RNA polymerase II transcripts [12, 13]. Genomic mapping has shown that pri-miRNAs are often derived from the intron of either protein-coding or noncoding RNA (ncRNA) transcripts and less frequently from the exon of ncRNAs [14]. Within the nucleus, the pri-miRNA forms a stem-loop structure that is recognized by the microprocessor complex which contains two core components, the RNase III endonuclease *Drosha* and the double-stranded RNA binding protein *DGCR8* (Di George

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Syndrome critical region gene 8) [15, 16]. DGCR8 binds to the stem-loop structure and then guides Drosha into position, cleaving ~11 base pairs (bp) from the base of the stem-loop to produce a 70–100 bp hairpin RNA molecule designated the precursor miRNA (pre-miRNA) [17]. The pre-miRNA is subsequently transported from the nucleus by Exportin 5, a nuclear export receptor, to the cytoplasm [18, 19]. Once in the cytoplasm, a second RNase III endonuclease, Dicer, cleaves the pre-miRNA to produce ~22 nucleotide double-stranded RNA molecule in which one strand, the mature miRNA, is transferred to the RNA-induced silencing complex (RISC) containing Dicer, a member of the Argonaute protein family and the RNA binding protein Tarbp2 (TAR (HIV) RNA binding protein 2); the other strand is typically targeted for degradation [20]. The mature miRNA directs RISC to 3'-UTR of target mRNA through complementary binding of the miRNA seed sequence which results in inhibition of translation and/or degradation of the target transcript [21].

Tissue-specific expression

With the identification of new miRNAs came the realization that some miRNAs were not ubiquitously expressed as *let-7*, but instead appeared to have a restricted pattern of expression. For example, the mature form of miRNA-1 (miR-1) was found to be expressed only in the human heart but not in the brain, kidney, liver, lung or HeLa cells [8, 10]. The concept that some miRNAs were expressed in a tissue-specific fashion was confirmed in a study by Lagos-Quintana and coworkers (2002) showing that miR-1, -122a and -124a expression was restricted to striated muscle, liver and brain, respectively [11]. In a screen for new miRNAs, Sempere et al. (2004) identified 30 miRNAs that were enriched or specifically expressed within a particular tissue [22]. This study also provided the first description of striated muscle-specific miR-1, -133a and -206, which were later designated as myomiRs [22, 23]. The myomiR family has expanded to include miR-208a, miR-208b, miR-499 and, most recently, miR-486 (see Table 1) [24–26]. Northern blot analyses showed that these new members of the myomiR family are strictly striated muscle-specific (miR-208a, miR-208b and miR-499), being derived from the intron of different muscle-specific myosin heavy chain genes, or highly enriched in muscle (miR-486) [24, 25]. Most myomiR family members are expressed in both the heart and skeletal muscle except for miR-208a, which is cardiac-specific, and miR-206, which is skeletal muscle-specific and enriched in slow-twitch muscles such as the soleus (see Table 1) [27].

MyomiRs

From the time of their original description, much effort has gone into determining the function of myomiRs in striated muscle (reviewed in Liu and Olson, 2010) [28]. The finding that over-expression of miR-1 caused a shift toward a myogenic profile in HeLa cells suggested myomiRs may have a fundamental role in promoting a muscle cell identity [29]. The importance of myomiRs in myogenesis was first demonstrated by the finding of Sokol and Ambros (2005) who reported the deletion of miR-1 in the fly resulted in premature death from a failure of skeletal muscle to properly grow during early development [30]. Table 1 lists the known myomiRs, their host gene, expression pattern and whether or not they have been knocked out in the mouse. Interestingly, myomiRs appear to have either uniform expression throughout the muscle (miR-1 and miR-133a), independent of fiber-type, or are enriched in slow-twitch, type I muscles (miR-206, miR-208b and miR-499) [25,

27]; to date, no myomiR has been reported to be enriched specifically in fast-twitch, type II muscle. However, RNA-seq analysis of porcine and bovine skeletal muscle has identified non-myomiRs that are relatively enriched in a muscle of a particular fiber type [31, 32]. For instance, Muroya and colleagues reported that in steers miR-885 and miR-196a were highly enriched in the fast-twitch semitendinosus muscle relative to the slow-twitch masseter muscle [32].

As detailed in Table 1, most of the myomiRs have been deleted in the mouse with surprisingly little impact on skeletal muscle phenotype [25, 26, 33–37]. For example, deletion of skeletal muscle-specific miR-206 resulted in no obvious phenotype as reflected by no change in soleus muscle weight, morphology or fiber-type distribution; however, recovery from denervation was delayed in muscle of the miR-206 knockout [35]. In a similar manner, the Olson laboratory showed that miR-208a was necessary for the stress response involved in cardiac hypertrophy [26]. Collectively, these findings are consistent with the idea that a primary function of miRNAs is to mediate the stress response of the cell by helping to restore homeostasis through regulating gene expression [38]. It is not exactly clear why these knockout mice do not show a more dramatic phenotype as would be predicted from *in vitro* studies [39, 40]. One possibility is the overlap in gene targets between myomiR family members, such as miR-1 and miR-206, which could rescue any deleterious phenotype resulting from the miRNA knockout. This idea is supported by the double knockout of miR-133a-1 and miR-133a-2 in which mice showed septal defects and skeletal muscle myopathy that was not present in the single miR-133a knockout mice [41, 42].

The purpose of this review is to present what is currently known about the role of myomiRs, as well as other miRNAs, in skeletal muscle in response to both resistance and endurance exercise following either an acute bout or with training. The review will also cover the function of miRNAs in satellite cells, muscle injury and repair, aging and disease.

MicroRNAs in exercise

It is well established that exercise of sufficient intensity will place mechanical and/or metabolic stress on the contracting muscle. Accordingly, it is reasonable to speculate *a priori* that miRNAs will have a role in the stress response of skeletal muscle to changes in contractile activity. Defining the role of miRNAs in skeletal muscle plasticity is still in its infancy such that many of the exercise studies provide data that is purely correlative in nature. For the field to continue moving forward, future studies will need to provide more mechanistic data through the validation of punitive miRNA target genes and incorporate such genes into a regulatory network.

Exercise has repeatedly been shown to be a potent activator of gene expression, with expression patterns varying considerably depending on the mode of exercise [43]. Furthermore, exercise, particularly resistance exercise, results in the activation of various signaling cascades which in turn promotes an increase in protein synthesis [44–46]. Some of these alterations in gene expression may be attributable to changes in miRNA levels, as recent investigations have demonstrated that exercise induces changes in miRNA levels

within multiple cell types [47–49]. These fluctuations in miRNA levels may augment training adaptations by regulating specific genes involved in those adaptations (see Table 2).

Resistance Exercise and Skeletal Muscle miRNA Expression

Resistance exercise places a mechanical strain on the muscle resulting in a number of different adaptations, the most notable being a hypertrophy of the muscle; this hypertrophic growth occurs primarily through the accretion of contractile proteins thus increasing the capacity to generate greater force. A commonly used animal model of muscle hypertrophy is the synergist ablation model. This model involves the surgical removal of two primary synergist muscles thereby placing a mechanical overload on the remaining plantaris muscle and inducing a robust hypertrophy of the muscle. Following seven days of synergist ablation, expression of miR-1 and miR-133a were down-regulated by approximately 50% in the mouse plantaris muscle [27]. In humans, resistance exercise has been shown to modulate the expression of various miRNAs. In young men, an acute bout of resistance exercise coupled with ingestion of amino acids resulted in a significant decrease in miR-1 expression in skeletal muscle, in agreement with the loss of miR-1 expression in response to a hypertrophic stimulus observed in the mouse [27, 50]. This decrease in miR-1 expression following exercise may result in the modulation of protein synthesis, as miR-1 has been shown to directly target and inhibit various factors in the IGF1/AKT signaling pathway [51]. Though speculative at this time, it is thought that the changes in miRNA expression following an acute bout of resistance exercise are an integral aspect of the molecular changes underlying the adaptations observed following chronic resistance exercise training (see Figure 1).

In a study by Davidsen and colleagues (2011), a cohort of young, adult males underwent 12 weeks of resistance training with *post-hoc* analysis separating the group into “low-responders” and “high-responders” based on each subject's change in lean body mass [48]. The authors then examined whether expression levels of the most abundant miRNAs were different between the groups. Twenty-one miRNAs were profiled, showing that miRNA expression was unaffected in the vastus lateralis muscle in the high-responder group whereas the low-responder group showed a significant change in miR-451 and miR-378, with a downward trend for miR-26a and miR-29a. Interestingly, the change in miR-378 expression showed a significant correlation ($R^2=0.51$) to the change in lean body mass, leading the authors to speculate that maintenance of miR-378 levels were required for changes in lean body mass [48]. This idea is supported by *in vitro* data that shows that miR-378 promotes myoblast differentiation by targeting *MyoR*, a negative regulator of the myogenic transcription factor *MyoD* [52, 53]. In humans, it has been suggested that this myogenic differentiation and addition of nuclei to existing skeletal muscle positively influence muscle hypertrophy [54]. In addition, recently miR-378 has been shown to control mitochondrial metabolism and bioenergetics through the regulation of *PGC1-β*, and in the context of exercise, would likely be a physiological response observed with endurance exercise [55]. Therefore, the blunted hypertrophic growth in “low responders” to resistance exercise may be that these individuals are predisposed to an aerobic phenotype relative to a hypertrophic phenotype, i.e., loss of miR-378 expression lead to the upregulation of *MyoR* and *PGC-1β* (see Figure 1).

Endurance Exercise and Skeletal Muscle miRNA Expression

In contrast to resistance exercise, endurance exercise generally brings about metabolic adaptations with little change in skeletal muscle mass [56–58]. These metabolic adaptations are characterized by an increase in mitochondria, a fiber-type shift to a more oxidative profile and an increase in capillary density. A number of studies have reported changes in miRNA expression following endurance exercise suggesting miRNAs may have a role in the metabolic adaptations that occur with this type of exercise. For example, rectus femoris muscle of mice subjected to a 90 minute bout of forced running showed an upregulation of miR-1, miR-181 and miR-107 and downregulation of miR-23, with the decrease in miR-23 corresponding in an increase in both the mRNA and protein levels of one of its predicted target genes, *PGC1- α* [59]. Similarly, Aoi et al. (2010) demonstrated that four weeks of running resulted in the downregulation of another miRNA predicted to target *PGC1- α* , miR-696 [60]. Consistent with regulation by miR-696, *PGC1- α* protein expression was elevated whereas *PGC1- α* mRNA levels were unchanged suggesting miR-696 was acting by blocking translation [60]. *PGC1- α* is thought to have an important role skeletal muscle adaptation to exercise as it has been shown to be involved in mitochondrial biogenesis coupled with the control of energy expenditure, including gluconeogenesis, glycolysis and fatty acid oxidation [61] (see Figure 2). The findings from these studies suggest that the mechanism of miRNA regulation of *PGC1- α* expression is different in response to an acute bout or chronic training [59, 60]. Given that miR-696 is only expressed in the mouse, it remains to be determined if a similar mechanism via a different miRNA is operative in human skeletal muscle in response to endurance exercise.

In humans, 60 minutes of endurance exercise (cycling) was reported to increase the expression of miR-1 and miR-133 in the vastus lateralis muscle in untrained individuals, whereas following 12 weeks of training the resting levels of miR-1, miR-133a, miR-133b and miR-206 were lower than pre-training. [62]. The training resulted in a blunted response to the acute bout, which showed no changes in miRNA levels after training. Similarly, Keller et al. (2011) demonstrated that 6 weeks of cycling decreased the expression of miR-1 and miR-133, along with miR-101 and miR-455 in human skeletal muscle [63]. These results suggest that miRNA expression levels may change as training status changes in order to regulate training adaptations; however, to provide a causative role for miRNAs in skeletal muscle adaptation to exercise will require the use of gain- and loss-of-function animal models.

In another mode of endurance exercise, Yamamoto et al. (2012) recently reported that an acute bout of swimming decreased the level of miR-494 in the gastrocnemius muscle of mice [64]. The authors went on to validate miR-494 regulation of two genes known to be involved in mitochondrial biogenesis, mtTFA and Foxj3. Another group examined miRNA levels in the soleus muscle of rats following swim training and found that miR-16 levels significantly decreased, paralleled by an increase in protein levels for *VEGF* and its respective type 2 receptor [65], with *VEGF* subsequently being validated as a miR-16 target by another research group [66]. These alterations within the VEGF pathway were thought to modulate angiogenesis, as there was a concomitant increase in capillarization within the muscle (see Figure 2). Collectively, the results of these studies suggest that miRNAs may

have a role in modulating those adaptations that underlie the increase in oxygen delivery and utilization that occur with endurance exercise.

Endurance Exercise and Cardiac Muscle miRNA Expression

Two of the primary determinates of aerobic capacity is oxygen delivery to the working skeletal muscle and the ability of the muscle to take up and utilize that oxygen. Therefore, changes must occur in the cardiovascular system in order to effectively deliver oxygen, some of which may be regulated by local expression of miRNAs.

In rats, 10 weeks of low-intensity swimming produced changes in miRNA expression within the left ventricle. Swimming produced decreased expression of miR-1, miR-133a and miR-133b, accompanied by an increase in the expression of miR-29c. This corresponded with a decrease in collagen type I and collagen type III, both known targets of miR-29c, which may be a possible mechanism relating the benefits of endurance exercise with ventricular compliance [49]. In addition, swim training increased the expression of miR-126 in the left ventricle promoting angiogenesis by regulation of repressive genes (*Spred-1*, *Pi3kr2*) within the vascular endothelial growth factor (*Vegf*) pathway [67]. Similarly, swimming increased miR-27a and miR-27b which targets angiotensin-converting enzyme (*Ace*), while decreasing miR-143 which targets *Ace2* in the heart [68]. Collectively, these adaptations would have a positive influence on cardiovascular function.

Exercise and MicroRNA Levels in the Circulation

In addition to striated muscle (heart and skeletal muscle), endurance exercise has also been shown to alter miRNA expression within the circulation. Plasma samples taken from human subjects showed that an acute bout of endurance exercise elevated circulating levels of miR-146a, miR-222, miR-21 and miR-221 [47]. Following a 90 day training period, the basal level of these miRNAs remained elevated in addition to miR-20a. Interestingly, even after the training period, circulating levels of these miRNAs still showed the transient increase following an acute bout of exercise [47]. Similarly, Aoi et al. (2013) recently showed that both acute and chronic endurance exercise decreased circulating levels of the muscle-enriched miR-486, however all other circulating myomiRs levels were below detectable limits [69]. The decrease of miR-486 levels in circulation may contribute to the negative protein balance typically associated with endurance exercise [70], as miR-486 suppresses factors that promote protein breakdown (*Foxo*) and inhibit protein synthesis (*Pten*) [71]. These studies all employed a candidate approach when assessing miRNA levels in circulation such that a more comprehensive screen may lead to the identification of additional miRNAs in circulation that are involved in the response and/or adaptation of muscle, and possibly other tissues, to exercise.

A recent study sought to determine if there was any relationship between circulating miRNAs levels and aerobic capacity [72]. The authors reported that individuals with a low aerobic capacity (VO_{2max}) had elevated levels of miR-21, miR-210 and miR-222; however, the elevated levels of these miRNAs in circulation did not show a correlation with risk factors associated with cardiovascular disease. In contrast, miRNAs do appear to be useful as a biomarker for exercise-induced muscle damage. Uhlemann and colleagues (2012) found

that circulating levels of miR-133a were elevated following an exercise bout designed to damage skeletal muscle, while miR-126 was elevated following exercise that would damage the endothelial layer within the vasculature [73]. The findings from these studies indicate that a bout of endurance exercise is capable of eliciting a change in serum miRNA levels, which are specific to the form of exercise, though the physiological implications remain to be understood. One possible mechanism may involve the release of miRNAs containing vesicles (known as exosomes) by the active skeletal muscle thereby acting as a paracrine factor influencing the activity of other tissues [74]. In addition, it is easy to imagine that circulating miRNAs may one day prove to be useful as a biomarker for assessing training status, or to serve as a predictor of disease progression (see Myopathies section).

A better understanding of how exercise, whether acute or chronic, is able to alter miRNA expression, will provide a more complete picture of the molecular mechanisms that underlie exercise-induced adaptations. This knowledge may offer insight into what training modalities are most beneficial, particularly in the context of disease prevention or treatment. Furthermore, miRNAs may themselves represent therapeutic targets that can be manipulated to augment training adaptations.

Muscle Atrophy

Disuse

In contrast to increased mechanical load, prolonged periods of mechanical unloading of skeletal muscle leads to muscle atrophy. This can occur as a result of prolonged bed rest [75], space flight [76] or denervation [75]. Allan and colleagues (2009) demonstrated that ~12 days of space flight produced a significant decrease in miR-206 expression in the mouse gastrocnemius muscle [76]. Using a rodent model of space flight, McCarthy and coworkers (2009) reported that seven days of hind limb unloading decreased miR-107, miR-221 and miR-499 expression; moreover, when unloading was carried out to 28 days, miR-499 was further decreased along with a 60% decrease in miR-208b [77]. Given that miR-499 and miR-208b are components of the myomiR network in skeletal muscle, these findings suggested the network is operative during skeletal muscle atrophy [25]. The authors provided evidence that supported the idea that miR-499 was involved in regulating the fiber-type shift associated with atrophy through the regulation of *Sox6*, a transcription factor known to repress slow myosin expression [78]. Studies have now shown that a miR-499/*Sox6* pathway acts as a master regulator in setting the fiber-type composition of a muscle [79, 80]. Unlike hind limb unloading induced muscle atrophy, following a month of denervation, the expression of miR-1 and miR-133a in the rat soleus was decreased by ~70% compared to sham control; surprisingly, after four month of denervation the expression of miR-1 and miR-133a was actually 2-fold higher than control [81]. This depression of miR-1 and miR-133 during an acute period of disuse was also observed following seven days of bed rest in the vastus lateralis of human subjects [82]. These findings indicate that skeletal muscle atrophy in both rodents and humans is associated with a loss of myomiR expression, though the mechanism of how these miRNAs may regulate atrophy remains to be resolved.

Aging

The change in gene expression in response to exercise has been shown to be significantly different in the elderly [83, 84]. This alteration in gene transcription may in part be modulated by differentially expressed miRNAs which have been shown to occur in aged skeletal muscle [85, 86]. When compared to younger individuals, skeletal muscle from older individuals showed a significant elevation in the miRNAs let-7b and let-7e under resting conditions, which was proposed to be involved in regulating the cell cycle based on let-7 target genes [85]. Older subjects also exhibited increased expression of primary miRNAs-1-1, -1-2, -133a-1 and -133a-2, although these changes did not correspond to an elevations in miRNA levels. Furthermore, following an acute bout of resistance exercise older subjects did not show the same decrease in miR-1 expression as observed in the young subjects [50]. Conversely, when older subjects participated in chronic resistance training, miR-1 levels were significantly decreased following the training period [87]. One explanation for the disparity between the findings of the two previous studies may be the timing of when the muscle was sampled post-exercise. The latest time point examined by Drummond et al. (2008) was 6 hours post-exercise, which could explain why they only saw a decrease in the primary miRNA and not the mature miRNA which could require a longer period or repeated exercise bouts, as used in the Mueller et al. (2011) study, before a significant decrease will be observed.

Muscle Function

One of the primary outcomes associated with chronic exercise is alterations in muscle function, however few investigations have examined what impact miRNA regulation of gene expression has on muscle function. In cell culture, Rhim et al., (2010) inhibited miR-133a activity and then bioengineered artificial skeletal muscle preparations [88]. Artificial muscle devoid of miR-133a showed a 20% increase in peak force that was paralleled by a greater myotube diameter relative to control myotubes [88].

Two of the primary motor proteins in skeletal muscle, α - and β -myosin heavy chain (MHC), have been shown to encode miR-208a and miR-208b, respectively. In addition, the *Myh7b* gene, a homolog of the β -MHC gene, is expressed in cardiac and slow-twitch muscles and encodes miR-499. Over-expression of miR-499, in conjunction with miR-208b, was shown to increase the number of type-I myofibers within a muscle. Consistent with the increase in type-I fibers, mice over-expressing miR-499 showed greater muscular endurance capacity as assessed by time to exhaustion with treadmill running [25]. Germline knockout of another myomiR, miR-206, however had no impact on downhill running time or distance [34]. Finally, over-expression of miR-1 had a negative effect on contractile function in cardiac muscle by disrupting sarcomere architecture, possible through targeting cardiac myosin light chain kinase and calmodulin expression [89]. These findings highlight the fact that manipulating the levels of a single myomiR may result in altered muscle function. Altering muscle function, particularly in cardiac muscle, can have deleterious outcomes, so any research aimed at potential clinical use of miRNAs needs to include some form of functional outcome measurement.

Satellite Cell Fate and Myogenesis

Satellite cells are mesenchymal stem cells within skeletal muscle that are located between the basal lamina and the sarcolemma. Under resting conditions these cells are normally quiescent, however, following an injury to the muscle they have the ability to re-enter the cell cycle, proliferate and either fused to an existing myofiber or fuse together to form a *de novo* myofiber; for a more comprehensive review on satellite cell dynamics the reader is referred to two recent reviews [90, 91]. A complex network of transcription factors control satellite cell quiescence, proliferation and differentiation. During quiescence, satellite cells express paired-box 7 (*Pax7*) transcription factor and following commitment to the myogenic lineage, express myogenic factor 5 (*Myf5*). Upon activation and proliferation, *Myf5* expression is up-regulated, along with another transcription factor, myogenic differentiation 1 (*MyoD*) [90]. Finally, during terminal differentiation *Pax7* is down-regulated and the transcription factor myogenin is up-regulated, along with muscle regulatory factor 4 (*MRF4*), both factors being required for terminal myogenic differentiation [90]. It should be noted that the most common cell line for studying myogenesis and satellite cells *in vitro*, is the C2C12 myogenic cell line which has been shown to mimic many of the same processes that occur *in vivo* [92].

Quiescence

Only recently has the molecular mechanism underlying satellite cell quiescence begun to emerge, revealing that quiescence is an active process that requires the integration of various signaling pathways [93]. Recently, Cheung and colleagues (2012) used a satellite-cell specific Dicer knockout to profile all of the miRNAs expressed in quiescent satellite cells [94]. These authors reported that miR-489 was required for maintaining quiescence by directly repressing the oncogene *Dek*, a protein required for re-entry into the cell cycle [94]. In addition to miR-489, miR-31 was found to contribute to satellite cell quiescence by suppressing the translation of *Myf5*, which was shown to be necessary for proliferation and differentiation of satellite cells [95]. In a recent study by Koning et al. (2012) they demonstrated that there is a global downregulation of miRNAs in human satellite cells during quiescence compared to proliferation, in particular miR-106b, miR-25, miR-29c and miR-320c [96].

Proliferation

In addition to quiescence, miRNAs have also been shown to regulate the proliferation of satellite cells. In one of the earliest reports, Chen and coworkers (2006) showed that miR-133a was capable of promoting proliferation by repressing the expression of SRF (serum response factor), a transcription factor known to block cell proliferation [40]. Similar to miR-133a, miR-682 was found to be highly up-regulated during myoblast proliferation, both *in vitro* and *in vivo*, with inhibition of miR-682 activity resulting in reduced myoblast proliferation [97]. *In vitro*, miR-27a has been shown to be involved in promoting myoblast proliferation by targeting myostatin [98].

Differentiation

Numerous groups have investigated the role of miRNAs in myogenic differentiation and, to date, these studies have provided evidence showing that the myomiRs have an important role in this process. Kim et al., (2006) were one of the first groups to show that miR-206 was required for C2C12 myoblast differentiation, primarily through the regulation of DNA polymerase [39]. Similarly, miR-1 was found to promote myogenic differentiation by down-regulating histone deacetylase complex 4 (*HDAC4*) expression which led to the de-repression of follistatin expression, an antagonist to myostatin activity [40, 99]. In addition to miR-1, miR-206 and miR-29 were also shown to modulate the inhibitory effects of TGF β signaling on myogenic differentiation through altered *HDAC4* expression [100]. Specifically, Winbanks and colleagues (2011) reported that miR-206 and miR-29 were capable of inhibiting TGF- β signaling by repressing *Smad3* expression, which in turn resulted in the downregulation of *HDAC4* expression [100]. More recently, Dey and coworkers (2012) demonstrated that miR-26a repression of *Smad1* and *Smad4* expression, confirming the importance of inhibition of TGF- β signaling by miRNAs for myogenic differentiation [101]. MyomiRs miR-1 and miR-206 have also been found to promote myogenic differentiation by regulating the expression of *Pax7* [102]. Conversely, the inhibition of miR-1 and miR-206 activity by an anti-miR resulted in an increase in *Pax7* expression and cell proliferation [102]. *Pax7* has also been shown to be a direct target of the newest member of the myomiR family, miR-486, whose expression has been shown to be upregulated during differentiation [103].

Recently, Goljanek-Whysall et al. (2012) used microarray analysis to identify other punitive targets of miR-206 that could be involved in myogenesis. They identified and validated numerous targets of miR-206, including known myogenic factors *Meox2*, *RARB*, *Fzd7*, *MAP4K3*, *CLCN3* and *NFAT5*, and went on to demonstrate that downregulation of these factors was required for differentiation to proceed in C2C12 cells [104]. MyomiR-133a has been shown to promote differentiation by regulating the IGF-1 pathway through downregulation of the IGF-1 receptor [105]. Other roles for miR-133a have been shown to include promoting sarcomeric actin organization [106] and inhibiting brown-adipocyte formation [107]; although miR-133a was originally shown to have a role in regulating cell proliferation, these more recent studies demonstrate miR-133a controls numerous molecular events which appear to be context dependent. While the previous miRNAs have been shown to promote differentiation, miRNAs can also function to inhibit myogenic differentiation. Recently, Wang et al., (2012) showed that miR-23a inhibited differentiation by directly regulating the expression of myosin heavy chain genes, proteins required for contractile function in mature myofibers [108].

Regeneration

One of the defining characteristics of muscle is its remarkable ability to regenerate following injury. Based on the absolute requirement for satellite cell participation in regeneration many of the miRNAs discussed previously have been implicated in controlling regeneration [109–112]. However, significant injury to muscle has been shown to alter expression of a wide

number of miRNAs, many of which were not reported in the aforementioned satellite cell studies [113–115] (see Figure 3).

The majority of the investigations examining miRNAs involvement in satellite cells dynamics utilized *in vitro* models, whereas studies focused on skeletal muscle regeneration require the use of *in vivo* model. This requirement is an important distinction because it allows for a comparison to the *in vitro* findings and whether they are of any biological relevance, since the *in vivo* model takes into consideration the involvement of other cells type that could provide further regulation of miRNAs expressed in muscle cells. Such comparisons between *in vitro* and *in vivo* finding, however, must be tempered by the fact that the most common models of muscle regeneration induce supra-physiological levels of damage. Moreover, it is important to keep in mind that other cell types (macrophages), in addition to satellite cells, are necessary for adequate regeneration to take place and also may express specific miRNAs [116]. One of the first reports to show *in vivo* changes in miRNAs during regeneration observed an increase in miR-181 [115]. The authors showed that miR-181 was strongly upregulated during the late-phase of muscle regeneration and that it targeted *Hox-A11*, a negative regulator of *MyoD* and terminal differentiation. This concomitant increase in *MyoD* resulted in an increase in miR-1 expression, which in turn suppressed the action of *HDAC4* and *follistatin* [99]. In the early time points following cardiotoxin injury, miR-351 expression increased transiently, allowing for satellite cell proliferation by suppressing of *E2f3* expression, an inhibitor of cell cycle progression [113]. Following cardiotoxin injury, miR-206 is highly upregulated, suggesting a role in muscle regeneration, which is consistent with the impaired regeneration, reported in the miR-206 knockout mouse [34]. The increase in miR-206 expression is required to downregulate various genes including *Pax7*, *Notch3*, *IGFBP5* [34] and *Hmgb3* [117], all of which would be inhibitory to the differentiation process. In addition, the increase in miR-1 expression during regeneration further contributes to the downregulation of *Pax7* expression [102]. Similarly, miR-26a is upregulated following cardiotoxin-induced muscle injury and local knockdown in the tibialis anterior results in delayed regeneration [101]. In the same model, miR-125b is downregulated following injury resulting in increased expression of one of its direct targets, insulin-like growth factor two (IGF-II) [114]. *IGF-II* regulates myogenesis and the suppression of *IGF-II* expression by exogenous administration of miR-125 impairs regeneration [114]. Furthermore, during regeneration, increased expression of miR-133 is required to prevent satellite cells from adopting a brown adipogenic lineage [107].

With literature continuing to emerge defining the role of miRNAs in regeneration, additional studies are examining the effect of exogenous miRNAs administration on the regeneration process. This could have potential clinical implications as a future therapy following muscle injury. Nosaka and colleagues (2010) demonstrated that exogenous administration of miR-1, miR-133 and miR-206 mixture enhanced regeneration following laceration injury, by attenuating fibrosis formation, increasing myogenic factor expression and promoting angiogenesis [118].

Myopathies

Accumulating evidence is showing possible links between miRNA dysregulation and muscular disorders. The most commonly studied group of disorders related to muscle disease is muscular dystrophy. Muscular dystrophies have multiple etiologies, however individuals almost always present with muscle weakness and muscle loss, both resulting in impaired locomotion [119]. Eisenberg and colleagues (2007) were the first to profile the expression pattern of miRNA in a cohort of human subjects that had various types of muscular dystrophy [120]. They found that in the dystrophic group, there was a dysregulation in multiple miRNAs, with the majority of differences being an upregulation in miRNA expression. Interestingly, miR-146b, miR-221, miR-155, miR-214 and miR-222 were consistently dysregulated in almost all of the subjects. In a follow-up study by the same group, miR-486 expression was shown to be decreased in patients with Duchenne muscular dystrophy (DMD) and target members of the Akt/Pten signaling pathways [71]. In particular, miR-486 regulated platelet-derived growth factor receptor β (*Pdgfrb*) expression which was associated with a compromised regenerative response [71]. In the *mdx* mouse, an animal model DMD, miR-206 expression was significantly upregulated only in the diaphragm and not limb musculature [121]. In the same mouse model, muscle from *mdx* mice displayed a significant elevation in miR-31 which was shown to target the dystrophin gene, possible exacerbating the phenotype [122]. The reason for the discrepancy between the human and the mouse findings with respect to miRNA expression remain unknown, though later studies reported upregulation of miR-206 in muscle of both the *mdx* mouse and DMD patients [123, 124]. Recently, Cacchiarelli et al. [125] utilized a low-density array to profile miRNAs that were dysregulated in skeletal muscle of the *mdx* mice. They demonstrated that miR-1, miR-29, miR-30c, miR-133a and miR-206 were all differentially expressed in the gastrocnemius muscle, with the downregulation of miR-1 and miR-29 a direct result of reduced dystrophin/nNOS signaling leading to increased activity of HDAC2. The loss of miR-1 expression resulted in an increase in the level of glucose-6-phosphate dehydrogenase, a validated target, which negatively affected the redox state of the cell making it more susceptible to oxidative damage. Furthermore, loss of miR-29 expression was shown to promote fibrosis in *mdx* muscle [125].

ALS

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease that affects skeletal muscle and is characterized by loss of motor neurons, denervation of target muscles, muscle atrophy, and ultimately, paralysis [126]. Williams and colleagues (2008) demonstrated that miR-206 was drastically up-regulated in lower limb muscles of G93A-SOD1 transgenic mice, a model of ALS [35]. Inactivation of miR-206 revealed that miR-206 is involved in a retrograde mechanism that promotes reinnervation through downregulation of *HDAC4* [35]. This data is supported by a recent report looking at miRNA levels in muscle samples taken from human patients with ALS [127]. This group reported that in addition to miR-206, the expression of miR-23a, miR-29b, miR-31 and miR-455 was also upregulated in skeletal muscle from ALS patients. Of interest was the finding that miR-23a was shown to directly regulate *PGC1- α* , one of many genes important for mitochondrial function that was down-regulated in ALS patients. These findings suggest that the mitochondrial dysfunction

commonly observed in ALS patients may in part be mediated by mis-regulation of miR-23a, though this mechanisms remains to be tested [127].

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue sarcoma derived from skeletal muscle progenitor cells that continue to proliferate, having reduced capacity to differentiate into mature muscle cells [128, 129]. Given miRNAs clear role in the regulation of myogenesis, specifically differentiation, it seems plausible that miRNAs may play a role in development of specific types of RMS. The muscle-specific nature of the myomiRs made them obvious targets for potential deregulation in RMS. Support for this idea was first reported by Yan and coworkers (2009) who showed that in RMS cell lines and primary tumors miR-1 and miR-206 levels are repressed resulting in increased levels of the oncogene *c-Met*, a confirmed target gene [130]. Similarly, Roa et al., (2010) showed that miR-1, along with miR-133, were down-regulated in RMS cell lines and that this was paralleled by an upregulation in many of their predicted target genes [131]. These findings were confirmed in a large cohort of tissue from primary RMS tumors, showing that miR-1, miR-133a, miR-133b and miR-206 expression were all reduced when compared to healthy skeletal muscle levels [132]. Interestingly, there was a significant inverse relationship between miR-206 expression and survival along with metastasis at diagnosis. Since miR-206 is involved in repressing proliferation and inducing differentiation of myogenic progenitors, it is conceivable that this loss of miR-206 expression is aiding in the proliferative nature of tumor formation. This was demonstrated by overexpressing miR-206 in multiple RMS cell lines which resulted in reduced proliferation and cell-cycle progression, accompanied by increased apoptosis [132].

Non-myomiRs have also been shown to contribute to tumor formation. Wang and colleagues (2008) showed that epigenetic silencing of miR-29 by the NF- κ B pathway was maintained in a RMS cell line thereby impairing differentiation [133]. In addition, downregulation of miR-29 in two other RMS cell lines resulted in an increase in two target genes (*cyclin D2* and *E2F transcription factor 7*) both of which are involved in cell cycle progression [93]. Similarly, miR-26a has been shown to be down-regulated in both RMS cells line and primary tumors [134]. Recently, Sarver and colleagues (2010) reported that miR-183 is over-expressed in RMS and possessed oncogenic potential by targeting two tumor-suppressors, *EGR1* and *PTEN* [135].

MicroRNA Regulation of Oxidative Stress in Skeletal Muscle

The aforementioned studies have clearly demonstrated that exercise is able to induce changes in miRNA expression; however, the underlying mechanisms that control these changes remain to be defined. One plausible scenario is the change in miRNA expression in response to exercise is in part brought about by the change in the redox status of the muscle fiber. Exercise has been shown to dramatically alter the redox environment within muscle [136], therefore it is conceivable that some of the changes in miRNA expression are being driven by altered redox signaling in the cell. This idea is not without precedence, as there are many examples of redox-sensitive miRNAs in other cell types such as endothelial [137],

neurons [138] and cardiomyocytes [139]. Therefore, the oxidative stress brought about during exercise may be one mechanism that is able to induce changes in miRNA expression in skeletal muscle.

Similarly, microRNAs themselves may be functioning to alter the redox state of the cell. A recent investigation showed that miR-31 was decreased both after an acute bout of endurance exercise and following a short-term training period [140]. The authors showed that miR-31 targets the transcription factor nuclear respiratory factor 1 (*Nrf1*), a key transcription factor involved in regulating the expression of mitochondrial-related genes during periods of oxidative stress [141, 142]. Therefore, these results suggest that a decrease in miR-31 levels may act as a protective mechanism within the cell to maintain mitochondrial function during periods of oxidative stress, such as during endurance exercise. Alternatively, miRNA levels may lead to increased levels of oxidative stress within the muscle. One example of this would be the increased expression of miR-21 that is observed after a bout of endurance exercise [47], as well following a period of functional overload (McCarthy, unpublished data). MiR-21 has been shown to inhibit the conversion of superoxide to hydrogen peroxide by directly targeting SOD3 and TNF α [143]. While increasing reactive oxygen species (ROS) in the cell following exercise has been thought to lead to deleterious effects, recent evidence suggests that ROS generation actually promotes some of the desirable physiological adaptations associated with exercise [144, 145]. Overall, these studies support the idea that miRNAs may regulate and be regulated by oxidative stress following exercise in skeletal muscle, though this exciting possibility awaits experimental validation.

Conclusion

MiRNAs have emerged as important players in the regulation of gene expression, being involved in most biological processes examined to date. The concept that miRNAs are primarily involved in the stress response of the cell makes them ideal candidates for mediating the response of skeletal muscle to changes in contractile activity. Although the field is still in its infancy, the studies presented in this review offer great promise that miRNAs will have an important role in mediating the response of skeletal muscle to different forms of exercise. This optimism, however, is tempered by the recognition that the findings to date supporting a role for miRNAs in skeletal muscle plasticity remain for the most part correlative. The challenge for the field moving forward will be to move beyond these descriptive studies by using available genetic mouse models and anti-miR technology to perform gain- and loss-of-function studies. Moreover, to obtain a more complete understanding of the molecular mechanism through which miRNAs regulate skeletal muscle gene expression in response to exercise, it will be essential to identify and validate predicted target genes.

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- MicroRNA expression changes in response to both acute and chronic exercise.
- Expression of muscle-specific microRNAs known as myomiRs change in response to exercise.
- The microRNAs involved in the adaptation to exercise appear to be specific for the type and intensity of exercise.
- MicroRNAs have a role in skeletal muscle development and muscle diseases such as muscular dystrophy.

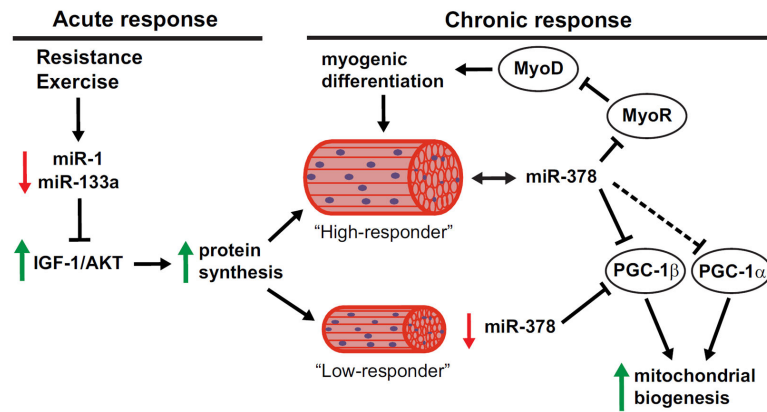


Figure 1. MicroRNA regulation of resistance exercise adaptations

Acute resistance exercise decreases miR-1 in skeletal muscle, leading to increased IGF1/AKT signaling and therefore, increased protein synthesis. In response to chronic resistance exercise, low-responders showed a decrease in miR-378 expression, a miRNA shown to regulate mitochondrial biogenesis via upregulation of *PGC-1 β* expression. Conversely, the high responders maintained their levels of miR-378, which is proposed to regulate myogenic differentiation by increased *MyoD* expression via repression of *MyoR* expression. Red arrow indicates downregulation of miRNA or gene expression. Solid line indicates validated gene target of miRNA, while dashed line indicates predicted gene target of miRNA.

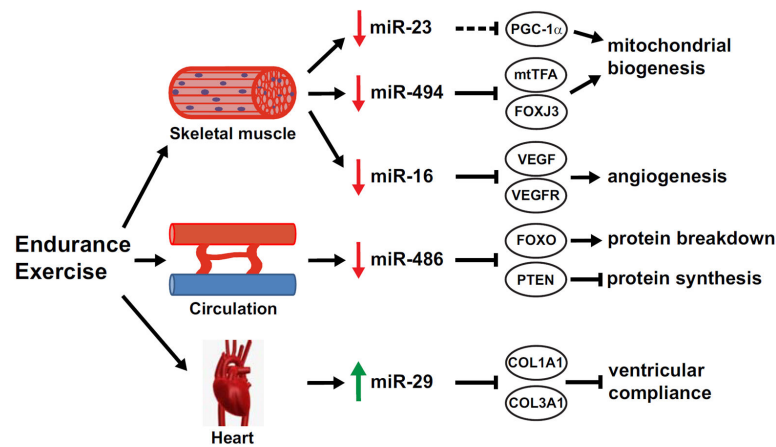


Figure 2. MicroRNA regulation of endurance exercise adaptations

Endurance exercise results in the downregulation of miR-23 and miR-494 expression in skeletal muscle which is predicted to promote mitochondrial biogenesis via upregulation of *PGC-1 α* , *mtTFA* and *Foxj3*. In Addition, miR-19 is downregulated which is predicted in increase angiogenesis by upregulation of *Vegf* and *Vegfr*. Endurance exercise decreases circulating levels of miR-486 which could promote a negative protein balance via regulation of *Pten* and *Foxo*. Finally, miR-29 levels are increased in the left ventricle following endurance exercise which is proposed to increase ventricular compliance by repressing multiple collagens. Green arrow indicates upregulation of miRNA or gene expression. Red arrow indicates downregulation of miRNA or gene expression. Solid line indicates validated gene target of miRNA, while dashed line indicates predicted gene target of microRNA.

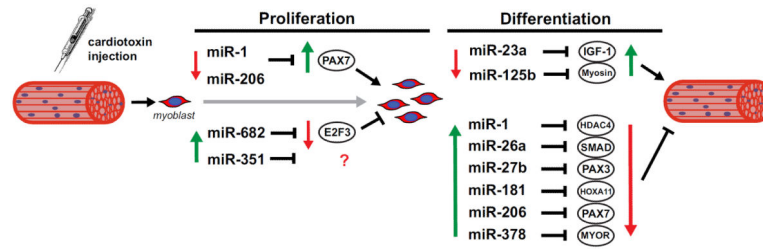


Figure 3. MicroRNAs and target genes involved in muscle regeneration

In response to cardiotoxin injury, activated satellite cells begin to proliferate and then proceed through myogenic differentiation as part of the regenerative process. Satellite cell proliferation and differentiation are associated with changes in miRNA expression that result in an alteration in target gene expression that have been shown to be important for proper muscle regeneration. Green arrow indicates upregulation of miRNA or gene expression. Red arrow indicates downregulation of miRNA or gene expression.

Table 1

Members of the MyomiR family of muscle-specific microRNAs.

myomiR(mouse)	Expression pattern	Host gene	Knockout phenotype	Reference
miR-1a-1	heart, skeletal muscle	intergenic	no knockout	■
miR-1a-2	heart, skeletal muscle	intergenic	50% lethal, cardiac defect	36
miR-133a-1	heart, skeletal muscle	intergenic	no overt phenotype	41
miR-133a-2	heart, skeletal muscle	intergenic	no overt phenotype	41
miR-133a dKO			septal defect, myopathy	41, 42
miR-133b	skeletal muscle, nerve	intergenic	no overt phenotype	37
miR-206	skeletal muscle (type I)	intergenic	no overt phenotype	35
miR-208a	heart	Myh6	blunted stress response	26
			conduction defects	33
miR-208b	heart, skeletal muscle (type I)	Myh7	no overt phenotype	25
miR-486	heart, skeletal muscle	Ank1	no knockout	■
miR-499	heart, skeletal muscle (type I)	Myh7b/14	no overt phenotype	25

Note. dKO, double knockout of miR-133a-1 and miR-133a-2.

Table 2

The effect of exercise on microRNA expression.

Mode of Exercise	Species	microRNA expression	Target gene	Outcome	Ref
<i>Resistance Exercise</i>					
Functional overload (c)	m	↓miR-1,-133a	IGF-1, IGF-1R	hypertrophy	27
Traditional RE (a)	h	↓miR-1	IGF-1, IGF-1R	hypertrophy	50
Traditional RE (c)	h	↓miR-378 ↑miR-451	growth pathways	low response to RE	48
<i>Endurance Exercise</i>					
Running (a)	m	↓miR-23 ↑miR-1, -181, -107	PGC-1α [*]	mito. biogenesis	59
Running (c)	m	↓miR-696	PGC-1α [*]	mito. biogenesis	60
Cycling (a)	h	↑miR-1, -133	MAPK & TGF-β pathways	modulate response to training	62
Cycling (c)	h	↓miR-1, -133a, -133b, -206			
Cycling (c)	h	↓miR-1, -101, -133, -455	RUNX [*] , SOX9 [*] , PAX3 [*]	angiogenesis	63
Swimming (a)	m	↓miR-494	mtTFA, FOXJ3	mito. biogenesis	64
Swimming (c)	r	↓miR-1, -133, -133b ↑miR-29c	COL1A1, COL3A1	incr. compliance	49
Swimming (c)	r	↑miR-129	SPRED-1, PI3KR2	angiogenesis	67
Swimming (c)	r	↓miR-143 ↑miR-27a, -27b	ACE, ACE2	hypertrophy	65
Swimming (c)	r	↓miR-16	VEGF, VEGFR2	angiogenesis	68

Note: a, acute; c, chronic; m, mouse; h, human; r, rat; mito, mitochondrial; incr, increase;

* predicted target.