REVIEW

microRNAs in skeletal muscle differentiation and disease

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ABSTRACT

miRNAs (microRNAs) are novel post-transcriptional regulators of gene expression. Several miRNAs, expressed exclusively in muscle, play important roles during muscle development, growth and regeneration; other ubiquitously expressed miRNAs are also essential for muscle function. In the present review, we outline the miRNAs involved in embryonic muscle development and those that have been found to be dysregulated in diseases associated with skeletal muscle or are changed during muscle adaptation. miRNAs are promising biomarkers and candidates for potential therapeutic intervention. We discuss the strategies that aim to develop novel therapies through modulating miRNA activity. In time, some of these approaches may become available to treat muscle-associated diseases.

INTRODUCTION

MicroRNAs (miRNAs or miRs) were first discovered in the nematode Caenorhabditis elegans, where a small non-coding RNA was shown to control the timing of cell differentiation during development [1]. Subsequently, miRNAs have been found to be widespread in both animals and plants, and have revolutionized our understanding of the regulation of gene expression.

miRNAs are approximately 22-bp non-coding RNAs found in most cell types. They normally act as negative regulators of gene expression, although it has been reported that miRNAs can activate gene expression in some instances [2,3]. Inhibition of gene expression is mediated by miRNA binding to sites often found in the 3′-UTR (untranslated region) of their target mRNA. This interaction leads to either degradation or repression of translation of the target mRNA depending on the degree of complementarity.

Production of miRNAs

Many miRNAs are generated from longer precursor RNAs in the nucleus. Longer non-coding precursor molecules, pri-miRNAs (primary miRNAs), are generated which contain characteristic stem-loop or

Key words: microRNA, muscle-associated disease, muscle-specific miRNA (myomiR), muscular dystrophy, non-coding RNA, skeletal muscle.

Abbreviations: ALS, amyotrophic lateral sclerosis; BAM, bioartificial muscle; BMD, Becker muscular dystrophy; ceRNA, competing endogenous RNA; CXMDj, canine X-linked muscular dystrophy in Japan; DM1, myotonic dystrophy type 1; DMD, Duchenne muscular dystrophy; FSHD, facioscapulohumeral muscular dystrophy; HDAC4, histone deacetylase 4; IGF, insulin-like growth factor; LNA, locked-nucleic acid; MEF, myocyte enhancer factor; miRNA (miR), microRNA; MRF, myogenic regulatory factor; MyoD etc., myogenic differentiation factor D etc.; myomiR, muscle-specific miRNA; Pax, paired box gene; PNA, peptide nucleic acid; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; RMS, rhabdomyosarcoma(s); TA, tibialis anterior; TGF-β, transforming growth factor-β; UTR, untranslated region.

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Figure 1 Biosynthesis of miRNAs and their mechanism of action
See the text for details.


hairpin structures. These generally have imperfect complementarity in the stem region and thus contain mismatches and bulges, rather than a perfect stem–loop. Hairpin structures can also be formed and processed into miRNAs from introns of coding genes [4]. These hairpins are released from the pri-miRNA by the microprocessor complex, which contains the dsRNA (double-stranded RNA)-binding protein DGCR8 (DiGeorge critical region 8) and the RNAse III enzyme Drosha [5–7]. At this stage, the resulting stem–loop structures of approximately 80 bp are known as pre-miRNAs. In general the structural characteristics of pri-miRNAs outside the hairpin are not well characterized [8], although it is known that sequences flanking the hairpin are important for processing, presumably to allow access of the microprocessor complex [9]. In addition, Drosha activity is dependent on the sequence of the pri-miRNA, and not all pri-miRNAs are processed with equal efficiency. Differential processing of these primary miRNA transcripts to pre-miRNAs provides a mechanism for regulating the relative amounts produced of mature miRNAs from their precursors [10,11].

One surprising aspect of the regulation of pri-miRNA processing has been the discovery that some transcription factors can also bind pri-miRNAs and affect their processing. These include the SMAD proteins, the effectors of TGF-β (transforming growth factor-β)/BMP (bone morphogenetic protein) signalling, which have been shown to bind to a range of pri-miRNAs to control their processing by Drosha [12,13]. Likewise, Drosha activity can also be regulated in cancer cell lines by interaction with the tumour suppressor protein p53 [14] and, in mice, the oestrogen receptor α can post-translationally regulate oestrogen target genes by regulating miRNA production [15]. These activities provide a direct link between cell signalling and miRNA production, and the control of Drosha activity by signalling molecules may well represent a general mechanism for miRNA regulation.

Another source of pre-miRNAs is derived from the RNA splicing machinery of the cell. In these cases, excision of a small intron from a longer coding RNA leads to the production of a small RNA capable of folding into a stem–loop known as a mirtron [16,17]. These miRNAs do not require the microprocessor complex, as they are generated directly from the splicing event. Other pathways have also been reported to result in production of miRNAs from various endogenous RNAs, such as snoRNAs (small nucleolar RNAs) and tRNAs (transfer RNAs) (reviewed in [18]).

Together, these various mechanisms are able to generate the characteristic RNA hairpins that are the basis for miRNA production. Following the generation of RNA hairpins, the separate pathways converge to generate the mature miRNAs.

Once the pre-miRNA is generated, it is transported from the nucleus to the cytoplasm for further processing to a mature miRNA. The first critical step is the removal of the loop to produce an RNA duplex. This essential step in miRNA maturation is carried out by another RNAse III enzyme, namely Dicer [19,20]. At this point there are two potentially functional miRNAs produced, referred to as the 5p and 3p miRNAs, depending on which end of the pre-miRNA they are derived from. Once the loop of the pre-miRNA has been removed, the two strands of the miRNA are unwound by RNA helicases and incorporated into the RISC (RNA-induced silencing complex) (Figure 1). Generally one strand is incorporated at much higher efficiency than the other [21,22] and, until recently, it was thought that only one of these products was functional and that the other, referred to as the miRNA*, was degraded. However, it has become clear that, in many cases, these less-abundant short RNAs are still functional [23] and, in some cases, the dominant
Mechanism of action of miRNAs

The RISC contains several proteins, including the Argonaute proteins AGO1–AGO4 [26]. They represent another critical point in the miRNA pathway at which miRNA function can be regulated. It is here that the decision is made about which of the miRNA strands, 5′ or 3′, will be loaded into the RISC. The Argonaute proteins have distinct expression patterns and activities, and determine the specificity and functionality of the miRNA [27]. They are also crucial for bringing the miRNA to its target sites in mRNAs.

The final step in miRNA-mediated gene regulation is the interaction of the miRNA with its target mRNAs. This is governed by the degree of complementarity between the miRNA and the target sequence, usually found in the 3′-UTR of the mRNA. In plants, most of the time miRNA and target sites are exactly complementary, making the identification of miRNA targets relatively straightforward; however, in animal systems, there is only partial complementarity between miRNAs and their target sequences and this makes the prediction of miRNA target genes more challenging. Although the rules governing miRNA–target interactions are not completely understood, it is clear that high levels of complementarity between residues 2 and 8 at the 5′-end of the miRNA and its target are required, leading to this region being referred to as the ‘seed’ sequence [28,29]. However, the seed sequence alone is not sufficient to define a miRNA target, as other more 3′ nucleotides of the miRNA also have an important role and some level of complementarity must exist with this 3′ region and the target site, although the details of this may vary according to each miRNA–target interaction [30]. Various algorithms have been developed to predict miRNA targets, leading to a range of target prediction databases, including PicTar [31], TargetScanS [32–34] and miRanda [25]. Because these and other algorithms can produce quite different lists of predicted targets, it is clear that more experimental evidence is required to identify crucial parameters and to improve target identification [29,35].

A number of experimental approaches have been developed to (i) validate miRNA–target interactions or to (ii) directly identify mRNAs that are associated with RISC. These include luciferase reporter constructs with potential target sites cloned into the 3′-UTR [36,37], direct pull-downs of miRNAs associated with their targets [38–40] and large-scale genomic screens [41,42]. An approach we have used recently to successfully identify miRNA targets is to compare the outputs of prediction algorithms and test those mRNAs predicted by multiple databases or known to have a biological role in the tissue of interest [43]. This can dramatically reduce the number of false positives, a major problem when faced with hundreds of possible targets to be tested. Although this almost certainly leads to the rejection of bona fide targets, this approach does provide an initial method of narrowing the list of genes to be analysed.

A high degree of complementarity between the miRNA and the target sites leads to degradation of the target mRNA and thus negative regulation of gene expression. This mechanism also seems to apply when synthetic siRNAs (small interfering RNAs) designed with complete complementarity mediate gene knockdown. However, in animal cells, the level of identity between the miRNA and the target site is usually less than 100% and, in some of these cases, mRNAs are not degraded but instead translation of protein is repressed [28,44]. It remains unclear which of these is the dominant mechanism, and different approaches have identified the primary effects as being mRNA degradation [45] or repression of protein production [46], whereas other groups have reported a combination of both these mechanisms [47]. It has also been suggested that in fact both degradation and repression are different stages of the same mechanism for controlling gene expression where initial repression of translation is followed by mRNA degradation [48]. Our recent findings suggest that the balance between the effects on mRNA stability and repression of protein synthesis varies depending on the individual target. Different targets seem to respond with a distinct combination of mRNA- and protein-mediated effects, even in the same cell type [43]. It may be the case that determinants of miRNA activity on a potential target site extend beyond the target sequence itself and include surrounding sequences in the 3′-UTR. Furthermore, many transcripts contain multiple putative miRNA target sites and thus are potentially under the control of more than one miRNA. This raises the possibility that closely positioned target sites may lead to synergistic or antagonistic effects, resulting in potentially very complex regulatory networks and possibly different responses in different tissues. The resolution of these questions will require the identification and detailed analysis of many miRNA targets, and it is possible that it is the cellular and molecular context that determines the outcome rather than just the interaction between the miRNA and the target site.

General function of miRNAs in cell differentiation

A major question is how influential the effects of miRNA expression are. This is often framed in terms
of whether miRNAs can act as major determinants in cell fate and behaviour [49] or if they are more subtly ‘fine-tuning’ gene expression. Another possible role of miRNAs is in buffering gene expression levels and thus canalizing development [50,51]. It has been predicted that most mammalian mRNAs are targeted by miRNAs [34] and some of the higher level gene regulatory networks can involve thousands of interactions of miRNAs and their targets [41], making them potentially very powerful regulators of cell behaviour. Recent work has demonstrated that co-ordinated miRNA regulation can target sets of functionally related mRNAs with the potential to radically change the genetic landscape of a cell [52]. A striking study has shown that the expression of just three miRNAs, miR-200c, miR-302 and miR-369, is able to induce pluripotency in mouse cells [53], showing a remarkable ability to entirely rewrite existing cellular programmes purely through miRNA expression.

Potential impact of miRNAs

miRNAs are curated and listed in miRBase, an online repository for validated miRNA sequences [25,54]. At the time of writing there are 21 643 different miRNAs across a range of animals, plants and viruses, of which 1921 have been identified in humans. The total number of miRNAs in the human genome is still a matter of speculation, although advances in high-throughput sequencing and prediction that most mammalian mRNAs are targeted by miRNAs [34] and some of the higher level gene regulatory networks can involve thousands of interactions of miRNAs and their targets [41], making them potentially very powerful regulators of cell behaviour. Recent work has demonstrated that co-ordinated miRNA regulation can target sets of functionally related mRNAs with the potential to radically change the genetic landscape of a cell [52]. A striking study has shown that the expression of just three miRNAs, miR-200c, miR-302 and miR-369, is able to induce pluripotency in mouse cells [53], showing a remarkable ability to entirely rewrite existing cellular programmes purely through miRNA expression.

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miRNAs IN MUSCLE DEVELOPMENT

The development of skeletal muscle provides an excellent paradigm for understanding the processes of cell fate and differentiation [58,59]. There are also important similarities between embryonic muscle development and muscle regeneration in adults, making developmental studies relevant to potential clinical applications [60]. As a well-characterized developmental system, it has also been extremely valuable in understanding the functions of miRNAs. Several lines of evidence have shown powerful effects of miRNAs in muscle development. In mice, Dicer has been genetically removed specifically in muscle cells and zebrafish with a loss-of-function mutation in Dicer have also been produced. These animals, which produce no miRNAs in muscles, show abnormal muscle fibre development and reduced muscle mass [61,62].

myomiRs (muscle-specific miRNAs)

One particular group of miRNAs, the myomiRs, have been studied extensively and have provided some important insights into how these molecules can regulate the myogenic programme. myomiRs are highly and specifically expressed during cardiac and skeletal muscle cell differentiation [63,64], and their functions are conserved from vertebrates to invertebrate species, including Drosophila [65] and the nematode C. elegans [66].

myomiRs are the members of the miR-1/206 and the miR-133 families. miR-1 and miR-206 are closely related and have identical seed sequences, although there are three nucleotide differences in the 3′ region between miR-1 and miR-206. In humans and mice, there are three loci encoding these miRNAs, each of which contains one member of the miR-1/206 group and one member of the miR-133 family. miR-1-1 and miR-133a-2 are clustered on chromosome 20 in humans and chromosome 2 in mice, and miR-1-2 and miR-133a-1 are on chromosome 18 in both humans and mice, whereas miR-206 and miR-133b are found on chromosome 6 in humans and chromosome 1 in mice. Although these loci are spread throughout the genome, the mature miRNAs produced are very similar. miR-1-1 and 1-2 produce identical mature miRNAs as do the miR-133a-1 and miR-133a-2 loci. miR-133b differs from these by a single nucleotide at the 3′-end. The myomiRs are expressed in skeletal muscle and miR-206 is restricted to this cell type, whereas miR-1 and miR-133 are also expressed in the heart, where they regulate cardiomyocyte differentiation and heart morphogenesis [67].

The expression of these miRNAs has been examined in detail. They are up-regulated during the early stages of muscle differentiation in both cell culture models [68–71] and in developing embryos [63,72,73]. Expression of these miRNAs is directly regulated by MRFs (myogenic regulatory factors), which are master regulators of muscle development, typified by the MyoD (myogenic differentiation factor D) bHLH (basic helix–loop–helix) transcription factor [67,74]. Although these miRNAs are closely linked and share at least some regulatory elements, the expression of precursor RNAs at this locus is complex. miR-1/206 and the corresponding miR-133 are not always produced from a single pri-miRNA encoding both miRNAs [75,76], and there are distinct promoters and splicing events that regulate the production of the two miRNAs at these loci.
miRNAs in cellular models of myogenesis

Interestingly, cell culture models of myogenesis have shown that miR-1/206 and miR-133 appear to have opposite effects in muscle differentiation. When overexpressed in cultured myoblasts, miR-1/206 promotes differentiation, whereas miR-133 opposes it by promoting myoblast proliferation, at least in part by down-regulation of serum response factor [69]. These opposite effects are mediated by distinct mRNA targets. miR-1/206 targets include follistatin and utrophin, both of which act to maintain myoblasts in a proliferative state [71]. Other validated targets include DNA polymerase a [70], HDAC4 (histone deacetylase 4) [69], connexin 43 [68], calmodulin and MEF2A (myocyte enhancer factor 2A) [77]. A recent screen in C2C12 myoblasts identified nine novel targets for miR-1/206, which, when overexpressed, caused the expression of genes related to alternative cell fates, specifically osteogenesis and chondrogenesis [43]. This illustrates that orchestrated regulation of multiple targets by myomiRs is not only important for the maintenance of the myogenic programme, but also crucial to prevent activation of non-muscle fates. In satellite cells, the adult stem cells of muscle, miR-1/206 can down-regulate the Pax3 and Pax7 genes (where Pax is paired box gene), both of which maintain a proliferative state [78–80]. In addition, the effects on Pax7 in these cells are mediated in part by another miRNA, miR-486 [79], whereas Pax3 is also targeted by miR-27 [81,82]. Microarray analysis has revealed that miR-489 functions as a regulator of satellite cell quiescence through the suppression of Dek, an oncogene that is asymmetrically inherited by the more differentiated daughter cell during cell division [83].

miR-1 is also regulated by IGF (insulin-like growth factor)-1, a well-known regulator of muscle growth and development. IGF-1 and its receptor are both predicted targets of miR-1 and also act as negative regulators of miR-1 expression. This generates a feedback loop between the IGF signalling pathway and miR-1 expression in muscle differentiation [84].

A fascinating twist to the regulation of myomiR function has been reported recently with the discovery that the pre-RNA which generates miR-133 can also act as a ceRNA (competing endogenous RNA). ceRNAs are non-coding RNAs that can bind to miRNAs in order to sequester them from their normal targets, and they have been proposed to have a major role in the regulation of miRNA function [85]. This non-coding RNA, linc-MD1, is expressed exclusively in muscle cells and binds to two miRNAs, miR-133 and miR-135. The sequestration of these miRNAs then leads to expression of muscle-specific genes such as MAML1 (mastermind-like 1), a miR-133 target, and MEF2C, a miR-135 target [75].

Other important miRNA regulators of muscle development include miR-24, which enhances myogenesis and is negatively regulated by TGF-β [86]. TGF-β signalling has also been shown to regulate miR-206 and miR-29 to alter the expression of HDAC4 [87]. Other examples include miR-181, which regulates HOXA11 (homeobox A11) during muscle differentiation [88], miR-221 and miR-222, which target the cell-cycle regulator p27 and can delay cell-cycle withdrawal during muscle cell differentiation [89], miR-214, which targets both EZH2 (enhancer of zeste homologue 2), part of the polycomb complex controlling epigenetic modifications of chromatin [90], and N-ras, the down-regulation of which facilitates cell-cycle exit [76], miR-378 which down-regulates MyoR (myogenic differentiation factor D), a repressor of myogenic differentiation [91], and miR-123b, which targets IGF-II, an important regulator of muscle growth.

Taken together, these findings described above (summarized in Figure 2) show the powerful effects on muscle cell differentiation mediated by miRNAs and provide clear evidence of miRNAs as core components of the myogenic differentiation programme.

Animal models of miRNA function in muscle growth and development

Several animal models have been used to analyse the effects of myomiR function. Texel sheep carry a single mutation in the 3’-UTR of their myostatin gene, which is required to limit muscle growth [92]. This G→A point mutation creates a target site for both miR-1 and miR-206 [93] and makes myostatin susceptible to miRNA-mediated repression; this is sufficient to lead to a hypermuscled phenotype [93,94]. The biochemical methods used to identify this novel miRNA regulation are readily applicable and, in combination with high-throughput sequencing approaches, could be extended to systematically screen for common and rare SNPs (single nucleotide polymorphisms) that might affect target gene regulation by miRNAs.

In zebrafish, loss of miR-1 and miR-133 leads to the disorganization of muscle segments and muscle gene expression [62]. Although mice lacking either one miR-1 locus [95] or miR-206 [96] form apparently normal skeletal muscle, we have shown that the transient inhibition of miR-206 in chick embryos leads to a delay in myogenesis [97]. This was, at least partly, mediated by the failure to fully down-regulate Pax3 in a timely fashion, a requirement for muscle differentiation [97]. The differences in these loss-of-function studies may be due to the redundancy of the three loci encoding the miR-1/206 alleles; in mice, miR-1 and miR-206 are expressed simultaneously, whereas, in chick embryos, the onset of miR-206 expression is earlier, thus the loss of miR-206 is not immediately compensated for by the expression of miR-1. Although the loss of miR-206 in mice does not affect embryonic muscle, it does have an effect in adult animals, where it promotes regeneration of neuromuscular synapses after nerve injury and can

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miRNAs known to be involved in skeletal muscle biology

For simplicity, embryonic myoblasts and satellite cells have been combined as there is significant overlap in their regulation. However, some interactions have not been shown to occur in both cell types. miRNAs which enhance differentiation are shown in blue, and miRNAs which repress differentiation are in red. See the text for details.

delay progression of a mouse model of ALS (amyotrophic lateral sclerosis) [96]. Gain-of-function studies have also shown strong effects on muscle development and, in *Xenopus* embryos, muscle segmentation and patterning was disrupted when either miR-1 or miR-133 was overexpressed [69].

Another aspect of miRNA function has been uncovered in the differentiation of fast- and slow-muscle fibres. At least three slow and cardiac muscle-specific MHC (myosin heavy chain) genes have been found to have miRNAs encoded in their introns. Loss of two of these miRNAs, miR-208b and miR-449, leads to the loss of slow-muscle fibres in the soleus muscle of mice, whereas overexpression of miR-449 can convert fast fibres into slow fibres in this muscle [98], illustrating the ability of miRNAs to affect cell fate choices in muscle lineages.

In addition to the above, high-throughput sequencing of short RNA libraries generated from developing somites has identified other potential miRNAs in this tissue [99]. Further analysis is required to determine whether some of these are involved in skeletal myogenesis and/or muscle pathology.

miRNAs are found and regulated throughout muscle development from the early proliferative myoblast to the mature differentiated muscle cell. Either loss or overexpression of miRNAs can have profound effects on myogenic programmes, indicating a significant role in the formation of muscle. The range of miRNA functions discovered so far in muscle biology strongly suggests that they are critical regulators of cell fate and development with a core function in cell biology. In the following sections, we will review the insights gained from studying some pathological conditions of skeletal muscle.

miRNAs AND SKELETAL MUSCLE DISEASE

Primary muscle disorders are caused by defective structural proteins, enzymes or abnormal signalling molecules and involve different groups of diseases, such as muscular dystrophies and myopathies. They are classified in accordance with their clinical and pathological manifestations. The major muscular disorders include the muscular dystrophies DMD (Duchenne muscular dystrophy), BMD (Becker muscular dystrophy), FSHD (facioscapulohumeral muscular dystrophy) and limb-girdle muscular dystrophies type 2A and 2B, as well as other myopathies, such as Miyoshi myopathy, nemaline myopathy, polymyositis, dermatomyositis and inclusion body myositis.

Comprehensive miRNA expression profiling has revealed that miRNA dysregulation is a common feature of muscle pathology (see Table 1). Microarray analyses revealed that 185 miRNAs are differentially expressed relative to controls in at least one of ten major muscle pathological conditions. A number of miRNAs were differentially expressed in just one disease, whereas miR-146b, miR-221, miR-155, miR-214 and miR-222 were dysregulated consistently in ten different diseases. Interestingly, these latter miRNAs are expressed in cells of the immune system, suggesting a likely association with inflammatory responses [100].

DMD and FSHD

As well as having a role in immune regulation, miR-222 has been found to be up-regulated in skeletal muscle of the *mdx* mouse, a model of DMD, where it participates in the down-regulation of β1-syntrophin, a component of the DPC (dystrophin–glycoprotein complex) in
miRNA abnormalities associated with skeletal muscle diseases in human tissue samples

<table>
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<tr>
<th>Disease/adaptive response</th>
<th>miRNA</th>
<th>Reference(s)</th>
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<tr>
<td>DMD</td>
<td>Increased expression of 37 miRNAs increased and 20 miRNAs decreased; increased expression of eight miRNAs including miR-206, decreased expression of miR-1, miR-135a and miR-29c; increased expression of miR-31 targeting dystrophin; decreased expression of miR-486</td>
<td>[2,100,102,104]</td>
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<tr>
<td>BMD</td>
<td>Increased expression of miR-221 and miR-146b</td>
<td>[100]</td>
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<tr>
<td>Limb-girdle muscular dystrophy type 2A</td>
<td>Increased expression of 80 miRNAs; decreased expression of miR-30a-3p and miR-197</td>
<td>[100]</td>
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<tr>
<td>Limb-girdle muscular dystrophy type 2B</td>
<td>Increased expression of 81 miRNAs; decreased expression of miR-30a-3p and miR-510</td>
<td>[100]</td>
</tr>
<tr>
<td>Miyoshi myopathy</td>
<td>Increased expression of 64 miRNAs; decreased expression of miR-30a-3p, miR-30c and miR-302c</td>
<td>[100]</td>
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<tr>
<td>FSHD</td>
<td>Increased expression of 57 miRNAs</td>
<td>[100]</td>
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<td>Polymyositis</td>
<td>Increased expression of 35 miRNAs; decreased expression of miR-30a-3p</td>
<td>[100]</td>
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<tr>
<td>Inclusion body myositis</td>
<td>Increased expression of 20 miRNAs; decreased expression of miR-197</td>
<td>[100]</td>
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<tr>
<td>Dermatomyositis</td>
<td>Increased expression of 33 miRNAs; decreased expression of miR-30a-3p</td>
<td>[100]</td>
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<tr>
<td>DMI</td>
<td>Increased expression of miR-206 in five out of seven patients; increased expression of miR-1 and miR-335; decreased expression of miR-29b, miR-29c and miR-33</td>
<td>[108,109]</td>
</tr>
<tr>
<td>RMS</td>
<td>Decreased levels of miR-1 and miR-132a; increased expression of miR-1, miR-133a, miR-133b and miR-206 in serum</td>
<td>[113,116,133]</td>
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<tr>
<td>ALS</td>
<td>Increased expression of miR-206</td>
<td>[96]</td>
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<tr>
<td>Atrophy</td>
<td>miR-1, miR-206, miR-133, miR-22a, miR-128, miR-499 and miR-208b are potentially protective</td>
<td>[32,117–120]</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>Expression of miR-206, miR-1, miR-133a, miR-23, miR-499, miR-378, miR-29a, miR-26a and miR-451 was found to be modulated</td>
<td>[98,117,121–124]</td>
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<tr>
<td>Growth and regeneration</td>
<td>myomiRs</td>
<td>[69,125]</td>
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dystrophic muscle [101]. Two independent studies have shown that miR-486, another muscle-enriched miRNA, is significantly reduced in patients with DMD, as well as in mdx mice [100,102]. Interestingly, miR-486 levels were not reduced in patients with BMD, who produce a partially functional dystrophin protein [100]. In primary myoblast cell cultures, inhibition of miR-486 resulted in reduced cell migration and failure to repair a scratch wound, whereas overexpression of miR-486 in these cultures resulted in increased proliferation. Specific overexpression of miR-486 in muscles of transgenic mice led to altered cell-cycle kinetics of regenerated myofibres in vivo. miR-486 is an important regulator of the PTEN (phosphatase and tensin homologue deleted on chromosome 10)/Akt pathway in dystrophin-deficient muscle and therefore an important factor in the regulation of DMD muscle pathology [102].

Further miRNA profiling using a real-time PCR platform revealed additional DMD-specific changes [103]. In that study, 11 miRNAs were found to be dysregulated in both mdx mice and DMD patients; among these, six had been shown previously to be dysregulated [100]. DMD-associated miRNAs were divided into three classes on the basis of their expression during the cycles of degeneration and regeneration or during the inflammatory response, indicating their potential involvement in different pathophysiological processes observed in diseased muscle. miR-222, miR-335, miR-29c and miR-206 were dysregulated in DMD, suggesting their involvement in the pathways involved in the muscle’s response to damage. Interestingly, six of these dysregulated miRNAs were also identified in the previous study [100]; three miRNAs, miR-29c, miR-222 and miR-335, had similar changes in expression, but the remaining three, miR-223, miR-449 and miR-206, were significantly increased in samples from DMD patients, although this was not been detected in the earlier microarray screen [100,103]. The differing results could be due to the greater sensitivity of real-time PCR and/or subtle differences in miRNA profiles between DMD patients with different mutations in the dystrophin gene. Notably, among the consistently up-regulated miRNAs were the muscle-specific miR-1 and miR-31.

Up-regulation of miR-31 in DMD samples has also been reported where it represses dystrophin expression; this suggests it may be part of a circuit controlling late muscle differentiation by repressing dystrophin and other markers. Consistent with this hypothesis, human DMD myoblasts that were treated with exon skipping showed increased dystrophin rescue following miR-31 inhibition [104].

miR-206 expression was significantly increased in the diaphragm of mdx mice, which is the most severely affected muscle in these dystrophin-deficient animals [105]. Increased expression of miR-206 has also been shown in regenerating and newly formed fibres in mdx mouse TA...
(tibialis anterior) muscles, but not in CXMDj (canine X-linked muscular dystrophy in Japan) TA muscles, which are derived from a canine model of DMD. The CXMDj muscles exhibited more severe and more progressive degenerative alterations than those seen in the mice [106]. Thus increased expression of miR-206 in mdx TA muscle is associated with active and efficient regeneration, whereas its reduced expression in CXMDj TA muscles correlates with severely impaired regeneration.

A possible involvement of miRNAs in FSHD has also been suggested [107]. FSHD, when occurring on a specific haplotype of 4qter (4qA161), is caused by deletion of a subset of the D4Z4 macrosatellite repeats in the subtelomeric region of chromosome 4q. Sense and antisense transcripts originating from the 4q D4Z4 units has been identified. Some of these generated small siRNA/miRNA-sized fragments; however, further studies are needed to confirm whether or not these are functional miRNAs.

**DM1 (myotonic dystrophy type I)**

DM1 is the most frequent autosomal-dominant myopathy in adults and is associated with the expansion of (CTG) triplet repeats in the 3′-UTR of the DMPK (dystrophy myotonica-protein kinase) gene. It is associated with myotonia, muscle wasting and weakness. Profiling of miRNA expression during myogenesis in muscle from patients affected by DM1 showed that, in the majority of these patients, only miR-206 was significantly overexpressed [108]. This is reminiscent of the observation from the diaphragm of mdx mice [105,108]. Interestingly, mRNA and protein levels of utrophin, an miR-206 target gene validated previously [71], were not significantly different between DM1 and control groups [108], also consistent with the studies in mdx mice [105]. A separate study examined the expression of 24 miRNAs in skeletal muscle biopsies from 15 DM1 patients and found that miR-1 and miR-335 were up-regulated, whereas miR-29b, miR-29c, and miR-33 were down-regulated compared with control samples [109]. The expression levels of predicted targets of miR-1 and miR-29 were also altered, suggesting that dysregulation of miRNAs was functionally relevant and could contribute to the pathogenesis of DM1. Both studies showed abnormal cellular localization of miR-206. In muscles from DM1 samples, miR-206 was prevalent in nuclear regions, mainly in nuclear clumps and centralized nuclei, whereas in cultured myogenic cells miR-206 was present in both the cytoplasm and nucleolus [108–110]. Although the localization of miR-206 in atrophic fibres from DM1 subjects might indicate its importance, it is not clear whether it is the cause or consequence of the pathological changes.

A recent study described MBNL1 (muscleblind-like splicing regulator 1), a protein implicated in DM1, as a regulator of pre-miR-1 biogenesis [110a]. In addition, that study showed that miR-1 processing is altered in heart samples from DM1 patients. As a consequence of the loss of miR-1, expression of GJA1 (gap junction protein, α1, 43kDa; connexin 43) and CACNA1C (calcium channel, voltage-dependent, L-type, α1C subunit; Cav1.2) were increased in hearts from both DM1 and DM2 patients, suggesting that miR-1 misregulation may contribute to the cardiac dysfunction observed.

**RMS (rhabdomyosarcoma)**

RMS is predominantly a paediatric sarcoma that resembles developing skeletal muscle and it accounts for over half of soft tissues sarcomas in children [111]. The presence of metastasis is associated with poor prognosis [112]. Current treatment is associated with significant toxicity, therefore there is a great demand for novel therapeutics.

Interestingly, miR-206 levels were shown to correlate with clinical behaviour of RMS. In general miR-206 expression levels were lower in RMS compared with skeletal muscle [113]. Low levels of miR-206 correlated with poor overall survival and shorter survival in metastatic embryonal and alveolar cases without PAX3/7-FOXO1 (forkhead box O1) fusion genes. In another study, miR-206 overexpression promoted myogenic differentiation of RMS cells by switching the global mRNA expression profile to one resembling mature muscle. This also blocked tumour growth in xenografted mice [114] and, in addition, miR-1 also promoted myogenic differentiation of RMS cell lines. miR-1/206 can also regulate cell proliferation and migration in RMS through targeting c-Met [115]. Similar to miR-206, the levels of miR-1 and miR-133a have also been shown to be drastically reduced in representative cell lines from major RMS subtypes [116]. The introduction of miR-1 and miR-133a into an embryonal RMS-derived cell line was cytostatic, suggesting a tumour-suppressor-like role for these miRNAs.

**ALS**

The expression of miR-206 has been shown to be elevated in a mouse model of the neurodegenerative disease ALS, and miR-206 has been identified as a key regulator of signalling between motor neurons and muscles [96]. Analyses of compound mutant mice showed that deficiency in miR-206 in the ALS mouse model accelerated disease progression and enhanced the degeneration of neuromuscular synapses. The protective effect of miR-206 in ALS was shown to involve the targeting of HDAC4. This in turn led to increased FGF (fibroblast growth factor) signalling pathways, thus promoting regeneration of neuromuscular synapses in response to motor neuron injury. The identification of miR-206 as a modifier of ALS pathogenesis suggests that the regulation of its levels could present a possible means of intervention in patients.
miRNAs related to muscle adaptation: atrophy and hypertrophy

miR-1, miR-206 and miR-133 have all been suggested to protect against atrophy in myocytes [117], as has miR-23a, which can suppress the translation of MAFbx (muscle atrophy F-box) [118], and miR-128, which has been theoretically predicted to target MDFI (MyoD family inhibitor) [32]. A bioinformatics approach suggests that miR-15a can reduce the atrophic effects of myostatin by down-regulating its receptor activin receptor-2B [32]. In a rat model of atrophy in soleus muscles, 18 miRNAs were significantly changed, including the significantly reduced expression of the muscle-specific miR-499 and miR-208b [119]. Interestingly, in this model, the expression of miR-1 and miR-133 was unchanged. This was similar to observations made in gastronomieus muscle of mice exposed to 12 days of spaceflight [120].

myomiRs have also been investigated in the context of muscle hypertrophy. pri-miR-206, but not mature miR-206, levels were increased in response to overload-induced muscle hypertrophy and the expression of miR-1 and miR-133a was decreased [121]. That study showed a role in promoting adaptation to overload by removing post-transcriptional repression of miRNA targets required for muscle growth. In human skeletal muscle, myomiR expression was significantly altered during post-exercise recovery [121]. Interestingly, this also included a change in the abundance of miRNA precursors, but not abundance of mature miRNA, when looking at the effects of exercise in young compared with old subjects [122]. Another study in young subjects showed that the expression of these miRNAs could be modulated following acute and chronic endurance exercise [123]. In mice, the expression levels of miR-1 and miR-23 changed following endurance exercise [117], and miR-499 has been shown to correlate with an increased muscle endurance [98]. A study in humans reported changes in a number of miRNAs following resistance exercise training. In particular, miR-378, miR-29a, miR-26a and miR-451 were differentially expressed between low- and high-responders and their expression correlated with the muscle mass gains observed in vivo [124]. myomiRs also play a role during muscle growth and regeneration [69,125], as shown in a rat skeletal muscle injury model, where injection of double-stranded miR-1, miR-133 and miR-206 accelerated muscle regeneration. For a recent review of miRNAs in muscle plasticity, also see [126].

miRNAs signatures as biomarkers of muscle diseases

miRNA signatures have recently been proposed as biomarkers for the detection of cancer, pregnancy and disease [127–129]. They may be a promising alternative for identifying disease, as well as monitoring treatment outcome and could, for example, replace the PCR sequencing methods currently used for the detection and diagnosis of genetic mutations causing myopathies [130].

Unique miRNA signatures that distinguish ten muscle diseases have been identified [100]. These signatures may provide a role for miRNA profiling in the diagnostic process. For example, on the basis of symptoms and significant up-regulation of miR-299, miR-5p, miR-487b and miR-362, a diagnosis of DMD, rather than BMD, could be made.

Creatine kinase blood levels, a measure of muscle damage, are utilized as diagnostic markers of several muscular diseases, including DMD. miR-1, miR-133 and miR-206 have been shown to be released into the bloodstream of DMD patients and their levels shown to correlate with disease severity. Increased serum levels of these miRNAs have also been found in mdx mice, and the levels are lowered in animals treated with exon skipping [131]. Another study has demonstrated that the increased serum levels of miR-1, miR-133a and miR-206 in mdx mice are affected less by exercise than creatine kinase levels [132]. Thus these muscle-specific miRNAs may present new stable biomarkers for the diagnosis of muscular dystrophy and potentially monitoring the outcomes of therapies. However, a larger collection of data from DMD patients is required to establish a precise correlation between miRNA levels and disease progression.

There is currently no serum biomarker of RMS, and muscle-specific miRNAs have been evaluated as a possibility [133]. Expression of myomiRs, especially miR-206, was significantly higher in RMS cell lines than in other tumour cell lines. Serum levels of miR-1, miR-133 and miR-206 were significantly higher in patients with RMS tumours compared with patients with non-RMS tumours, indicating that this could potentially be used for differential diagnosis.

Targeting miRNAs as Novel Therapeutic Approach

The sections above have indicated that aberrantly expressed miRNAs play key roles in disease, therefore restoring or antagonizing miRNA function could provide a method for therapeutic intervention. In the following section, we will summarize the current approaches that are being developed.

Antisense-based miRNA antagonists

This approach aims to inhibit miRNAs up-regulated under pathological conditions by using oligonucleotide sequences with complimentarity to endogenous miRNA.
These incorporate chemical modifications that enhance their ability to bind to and inactivate endogenous miRNA. These miRNA antagonists include anti-miRs, antagomiRs and LNAs (locked-nucleic acids). They inhibit function, as the blocked miRNAs can no longer enter the RISC or are degraded. Antisense targeting of miRNAs is an attractive approach due to the small size of the active molecule, and the therapeutic potential for miRNA antagonists has been suggested [134–136]. Early reports have shown that 2′-O-methyl-modified antisense oligonucleotides are capable of sequence-specific miRNA inhibition [137]. These inhibitors are resistant against nuclease degradation and have moderately strong miRNA inhibition. Improved anti-miRNA oligonucleotides have been reported, for example 2′-O-methoxyethyl-modified oligonucleotides [136]. LNA/DNA mixmers have also been shown to be very specific and effective in miRNA inhibition and these are now commercially available.

Inhibitors containing a cholesterol moiety conjugated to the 3′-end of antisense oligonucleotides, so-called antagomiRs [135], have been shown efficiently decrease miR-16 levels in mouse brains after local injection [138]. In addition, lentivirus-mediated expression of antagomiRs (and miRNA mimics; see below) can induce stable phenotypes for individual miRNAs [139]. These findings support the conclusion that antagomiRs could be powerful tools for studying miRNA function and that they represent potential therapeutics following targeted delivery in vivo.

PNAs (peptide nucleic acids) are charge-neutral oligonucleotide analogues that form strong sequence-specific complementary hybrids with RNA that can modulate gene expression in vitro by sterically block of essential RNA functions [140]. Lipofection of an LNA/2′-O-methyl mixmer antisense oligonucleotide or electroporation of a PNA oligomer has been effective in blocking miR-122 activity in human and rat liver cells [141]. These antisense oligonucleotides were more effective than standard 2′-O-methyl oligonucleotides in binding to and inhibiting miRNA action. CPP (cationic cell-penetrating peptide) PNAs, which enter cells through endocytosis, have been suggested as suitable for lead development in DMD [140]. However, further studies are needed to confirm the potential of PNA for future therapeutic applications.

In African green monkeys, an unconjugated, LNA-modified oligonucleotide (LNA-antimiR) can antagonize miR-122 in the liver [142]. Intravenous administration resulted in uptake of the LNA-antimiR in the cytoplasm of hepatocytes and the formation of stable duplexes with miR-122, leading to depleted miR-122 levels. As anticipated, this was accompanied by a long-lasting decrease in total plasma cholesterol. There was no evidence of LNA-associated toxicity or histological change, indicating the potential of LNA-antimiRs as a new class of therapeutics for diseases associated with miRNA dysregulation.

**miRNA sponges and target protectors**

As an alternative to chemically modified antisense oligonucleotides, transcripts containing multiple tandem-binding sites to a specific miRNA have been developed and are known as miRNA sponges [143]. These are conceptually equivalent to the natural cellular ceRNAs discussed above. Vectors encoding these miRNA sponges derepress miRNA targets after transient transfection. Their efficacy was comparable with chemically modified antisense oligonucleotides. A single sponge can be used to block an entire miRNA family that share the same seed sequence. Potentially, sponges directed against multiple miRNAs could be designed with numerous distinct binding sites for miRNAs. The authors envision the use of viral vectors or stably integrated transgenes to express sponges in animal models of disease and development. Continuous expression of miRNA sponges would enable long-term loss-of-function studies in cell-culture-based assays and in vivo, such as in cancer xenografts. Stable miRNA sponge activity has been achieved by expressing the transgene after chromosomal integration [144]. Achieving high levels of expression remains a challenge; however, recent reports have shown that partial miRNA inhibition can also yield informative phenotypes. Applications include the use of an adenoviral miRNA sponge to inhibit miR-133 in cardiac myocytes in a mouse model of cardiac hypertrophy [145] and an miR-31 sponge that was able to increase dystrophin synthesis 3-fold in DMD myoblasts when exon skipping was applied simultaneously [146]. Germline transmission of an miRNA sponge has been successful in Drosophila [147]; however, transgenic miRNA sponges in vertebrates have yet to be made to corroborate further the utility of this approach in vivo.

miRNA target protectors are stabilized antisense morpholino™ sequences (Genetools), which overlap with the target site. They are less widely used to block interactions between miRNA and a single target [97,148]. Target protectors are designed to prevent the interaction between an miRNA and a specific target, leaving other targets of that miRNA unaffected. This strategy could prove very useful in cases where a crucial disease-causing target can be identified or to prevent interactions between an miRNA and an aberrant target that is due to a mutation, which generates a target site, as for example in Texel sheep [93,94].

**miRNA mimics**

A practical approach to restore the levels of misregulated miRNAs has not yet been developed. However, miRNA mimics provide a potential alternative to gene therapy as they are substantially smaller than proteins or large DNA vectors, only need to enter the cytoplasm of
active cells and can be delivered systemically. Non-
specific off-target effects are not expected as the
mimics should recapitulate naturally existing miRNA–
rNA interactions. Consistent with this, transgenic
overexpression of miR-133a in skeletal muscle did not
result in any significant defects [149]. Because a single
miRNA can regulate a large number of target genes,
it has been proposed that restoring the expression of
miR-133 could restore the ‘cellular phenotype’ [150].
The therapeutic delivery of miRNA mimics raises the
question of toxicity in normal tissues and of possible
long-term effects for which in vivo evidence is still
lacking. However, some promising results have been
obtained in pre-clinical mouse models of cancer for a
small number of miRNAs. Introduction of a let-7 mimic
has been found to block the proliferation of cancer
cells and reduce the growth of lung tumours [151]. In
another example, systemic delivery of miR-133a
mimics could accelerate muscle regeneration
[152].

In a rat skeletal muscle injury model, it has been
demonstrated that local injection of miR-1, miR-206 and
miR-133 mimics could accelerate muscle regeneration
[125]. In these animals, the expression of the myogenic
markers, MyoD1, myogenin and Pax7 was induced after 1 week and combined application of miR-1, miR-133 and miR-206 promoted myotube differentiation. Thus local injection of miRNAs could be a promising novel therapeutic strategy in the treatment of muscle injury.

In DMD patients, a therapy involving intramuscular
injection of stabilized miR-486 along with exon-skipping
morpholinos has been proposed to restore muscle
function and prevent muscle loss observed in DMD
patients [102,153].

C-Met overexpression following the down-regulation
of miR-1/206 seems to be a common aetiology for
pathogenesis of RMS and miR-1/206 overexpression
could therefore provide therapeutic effects for RMS
patients by mediating cytotasis and promoting muscle
differentiation of poorly differentiated RMS cells [115].
miRNAs could also be considered as a supplement to
to chemotherapy, thereby reducing the toxicity associated with chemotherapy.

As miRNAs can regulate various cellular processes,
they present a promising approach to promote tissue
repair and regeneration. An interesting study examined
the effect of transient miRNA inhibition on the
function of three-dimensional skeletal muscle cultures
or BAMs (bioartificial muscles) [154]. The effects of
miRNA inhibition were assessed by functional force
measurements in response to electrical stimulation.
Transient transfection of anti-miR-133 into BAMs
promoted muscle differentiation and force production,
which indicates that modulation of miRNAs can have
functional effects in engineered tissues.

CONCLUSIONS

miRNAs are novel post-transcriptional regulators of gene
expression, and a combination of cell-based experiments,
animal studies and human studies have shown that they
play major roles in cellular differentiation programmes.
Several miRNAs, expressed exclusively in muscle, as
well as other ubiquitously expressed miRNAs, are
involved in muscle development, growth/adaptation and
regeneration. miRNAs are also essential for muscle
function and may have been found to be dysregulated
in diseases associated with skeletal muscle. This makes
miRNAs promising biomarkers and candidates for
potential therapeutic intervention. Many questions
remain; however, it is likely that novel therapies
modulating miRNA activity to treat muscle-associated
diseases will become available in the future.

ACKNOWLEDGEMENT

We apologize that, owing to space constraints, some
aspects of miRNA function in muscle biology could not
be covered in detail and some references had to be omitted

FUNDING

Our own work was supported by the Biotechnology and
Biological Sciences Research Council (BBSRC) [grant
numbers H019979, D016444].

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Received 7 December 2011/S April 2012; accepted 21 May 2012
Published on the Internet 1 August 2012. doi:10.1042/CS20110634

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