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MicroRNAs in skeletal myogenesis

Yejing Ge and Jie Chen*

Department of Cell and Developmental Biology; University of Illinois at Urbana-Champaign; Urbana, IL USA

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MicroRNAs (miRNAs) have emerged as critical regulators of numerous biological processes by modulating gene expression at the post-transcriptional level. It has become increasingly clear that almost all aspects of skeletal muscle development involve regulation by miRNAs. Many of these miRNAs have distinct expression profiles in skeletal muscles, under the regulation by the myogenic program. In the last few years the field has seen a rapid expansion of our knowledge of myogenic miRNAs that target a wide range of muscle genes to coordinately control the myogenic process. In this review we provide an up-to-date list of reported myogenic miRNAs and survey their expression patterns, regulation of biogenesis and gene targets in skeletal muscles. Emerging themes of miRNA regulation in the context of skeletal myogenesis will also be discussed.

MicroRNAs (miRNAs) are a class of evolutionally conserved non-coding RNAs of ~22 nucleotides, which regulate gene expression predominantly at the post-transcriptional level.[1] Eight years after the initial discovery of lin-4 in *C. elegans* in 1993,[2,3] dozens of miRNAs were reported simultaneously by several groups,[4-6] thus beginning the miRNA era. Over the last decade, hundreds of miRNAs were identified or predicted across a wide spectrum of species.[7] The prevalence and importance of this small RNA family in modulating gene expression has become increasingly clear. In this review, we will focus on one particular aspect of miRNA function—regulation of skeletal myogenesis. Intensive research activities in this area in recent years have led to the revelation that almost all aspects of skeletal muscle development involve regulation by miRNAs, with these myogenic miRNAs targeting a wide range of muscle genes to coordinately control the myogenic process. Here we will survey the myogenic miRNAs reported so far, and discuss the regulation of their expression in muscles, their gene targets and functions in skeletal myogenesis, and emerging themes of miRNA regulation.

Introduction: miRNA Biogenesis and Targeting

Mammalian miRNAs are encoded in the intergenic or intragenic (both intronic and exonic) regions of the genome, their genes often found clustered.[8] MiRNA genes are transcribed as long primary transcripts (pri-miRNAs), mainly by RNA polymerase II,[9] although polymerase III-dependent transcription has also been described in reference 10. Pri-miRNAs are processed co-transcriptionally by the nuclear RNase III Drosha/DGCR8 to generate ~70 bp pre-miRNAs, which are then exported by Exportin-5 to the cytoplasm in a Ran-GTP dependent manner. In the cytoplasm, pre-miRNAs are further processed by the RNase III Dicer to yield ~22 nt mature miRNAs (reviewed in ref. 9). The miRNA biogenesis paradigm has recently been expanded to include "mirtrons" that bypass Drosha processing,[11,12] and miRNAs (or miRNA-like small RNAs) that are generated independently of Dicer.[13-16] In addition, recent discoveries of secreted miRNAs in microvehicles communicating between cells further widen the scope of miRNA biogenesis and function.[17]

Through partial sequence complementarity, miRNAs exert their functions by binding to the 3'UTR of the target mRNAs[18] and subsequently directing them for translational inhibition and mRNA decay,[19] although translational activation by miRNAs has also been reported in reference 20 and 21. A most recent study has revealed that mammalian miRNAs inhibit gene expression predominantly through decreasing target mRNA levels.[22] It has been estimated that over 60% of the human protein-coding genes are under selective pressure to maintain miRNA sites.[23] With this enormous target repertoire, it is not surprising that miRNAs have emerged as key regulators for myriads of cellular and developmental processes. The early embryonic lethality of *Dicer* deficient mice and severe phenotypes from conditional *Dicer* knockout during different development stages and in various adult tissues,[24-27] attest to the pivotal roles of miRNAs.

Regulation of miRNA Expression in Skeletal Muscle

During skeletal muscle development, cells from the somites commit to myogenic lineage and progress along the myogenic pathway by proliferation, terminal differentiation and formation of multinucleated myofibers.[28] The activation of muscle specific transcription factors, including MyoD and MEF2 families of proteins, results in reprogramming of gene expression to govern skeletal myogenesis.[29,30] Modulation of myogenic gene expression by miRNAs has emerged as a new level of control for myogenesis. Mice with *Dicer* deleted in muscle die perinatally and display decreased skeletal muscle mass, increased apoptosis of the muscle cells, accompanied by abnormal myofiber morphology.[31] It is important to note that Dicer is also responsible for the production of other types of small RNAs, whereas DGCR8, the binding partner of Drosha, is specific to the miRNA biogenesis

*Correspondence to: Jie Chen; Email: jiechen@illinois.edu
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Table 1. Expression and targets of miRNAs known to function in skeletal myogenesis

microRNA	Tissue distribution	Expression upon myoblast differentiation	Targets in skeletal muscles
miR-1[a]	muscle-specific	Increase	HDAC4,[40] Cx43,[79] Pax7,[47] c-Met,[80] G6PD[84]
miR-24	ubiquitous	Increase	unknown
miR-26a	ubiquitous	Increase	Ezh2[38]
miR-27b	ubiquitous	Increase	Pax3[59]
miR-29b/c	ubiquitous	Increase	YY1,[54] COL1A1, ELN[84,94]
miR-125b	ubiquitous	Decrease	IGF-II[66]
miR-133	muscle-specific	Increase	SRF,[40] nPTB,[87] UCP2[88]
miR-181	ubiquitous	Increase	Hox-A11[58]
miR-206[a]	skeletal muscle-specific	Increase	DNA polα,[78] Fstl1,[35] Utrn,[35] Cx43,[79] TIMP3,[86] Pax7,[47,52,84] c-Met,[80,81] HDAC4[82]
miR-208b/499	muscle-specific	Increase	Sox6,[49] Purβ,[49] Sp3,[49] HP-1β[49]
miR-214	ubiquitous	Increase	Ezh2,[53] N-Ras[101]
miR-221/222	ubiquitous	Decrease	p27[61]
miR-322/424	ubiquitous	Increase	Cdc25A[60]
miR-486	muscle-enriched	Increase	FoxO1,[51] PTEN,[51] Pax7[52]
miR-503	ubiquitous	Increase	Cdc25A[60]

[a]miR-1 and miR-206 presumably share the same targets. Here we list the targets separately based on published experimental evidence.

pathway.[9] Of significance, striated muscle-specific *Dgcr8* knockout mice display severe dilated cardiomyopathy and heart failure,[32] although characterization of skeletal muscle in those mice has not been reported. Below we survey the expression of miRNAs with reported myogenic functions in skeletal muscle (**Table 1**).

MiR-1/miR-133/miR-206. The best-studied myogenic miRNAs are the miR-1/miR-206 and miR-133a/miR-133b families, consisting of four mature miRNAs expressed from three chromosomal loci as bicistronic transcripts.[33] These miRNAs are specifically expressed in cardiac and skeletal muscles under the control of the myogenic transcription factors SRF, MyoD and MEF2,[34-37] and they regulate the fundamental processes of skeletal myogenesis including myoblast/satellite cell proliferation and differentiation (reviewed in ref. 33). Among many miRNAs profiled, miR-1, miR-133 and miR-206 levels are most dramatically increased during myoblast differentiation.[38-40]

Recently, we have reported that miR-1 expression, under the control of its upstream and intragenic enhancers,[34,37] is regulated by mammalian target of rapamycin (mTOR) signaling.[39] This regulation is mediated by mTOR control of MyoD stability.[39] MyoD also regulates the expression of miR-133,[37] and miR-206,[35] and, as a key myogenic transcription factor, it is placed directly upstream of several other candidate myogenic miRNAs (discussed later). Interestingly, the results of our miRNA profiling suggest that the drastic increase of miR-133 and miR-206 levels during myoblast differentiation, like that of miR-1, is completely inhibited by the mTOR-specific inhibitor rapamycin.[39] In addition, several other miRNAs are induced during myogenic differentiation, albeit to lesser degrees, in a rapamycin-sensitive manner.[39] Hence, it is an intriguing possibility that the mTOR-MyoD axis may regulate a cohort of miRNAs in the coordinate regulation of skeletal myogenesis.

Adding to the complexity of regulation, miR-1, miR-133 and miR-206 have also been suggested to be controlled at the level of pri-miRNA processing. The RNA binding protein KSRP[41] is a component of both Drosha and Dicer complexes and it promotes the maturation of a subset of miRNA precursors through binding to their terminal loops.[42] MiR-1, miR-133 and miR-206 appear to belong to this subgroup of miRNAs. Future studies to examine the commonality of this regulatory mechanism in myogenic miRNAs should be illuminating.

The expression of these three well-studied myogenic miRNAs has also been examined in vivo. MiR-1,[39,43,44] miR-133,[43] and miR-206,[43-46] have all been reported to be upregulated during muscle regeneration in animals after injury. Interestingly, a transient drop in these miRNA levels has also been observed immediately after muscle injury.[43,44,46,47] Whereas this decrease may be a consequence of muscle damage and induction of fibrosis independent of muscle regeneration,[44] a recent report correlated this early phase of miR-1 and miR-206 suppression with their anti-proliferation effect on satellite cells.[47] miR-1 and miR-133a levels were also found decreased in skeletal muscles seven days after functional overload, accompanied by an increase in the corresponding pri-miRNAs,[48] implicating regulation at a miRNA maturation step. As the authors speculated, potential targets of these miRNAs may be involved in muscle growth regulation.[48] Alternatively, it is reasonable to suggest, based on the recent report,[47] that satellite cell activation and proliferation in response to the overload stimulus may require suppression of miR-1.

MiR-208/miR-499. Also displaying a muscle-restricted expression pattern is a family of intronic miRNAs named "MyomiRs,"[49] consisting of miR-208a, miR-208b and miR-499. Whereas miR-208a is restricted to cardiac muscle, miR-208b and miR-499 are expressed in both cardiac and skeletal muscles. These miRNAs are encoded by introns of their host myosin genes,

α-MHC, β-MHC and Myh7b, respectively, regulating myofiber type specification.[49] Interestingly, miR-499 was found to be the most dramatically decreased miRNA in a hind limb suspension-induced atrophy model, suggesting its potential importance in muscle maintenance.[50]

MiR-486. Another muscle-enriched miRNA is miR-486. Encoded in the gene Ankrin-1, the expression of miR-486 is activated by myocardin-related transcription factor-A (MRTF-A), SRF and MyoD.[51] Most recently, miR-486 is reported to be highly induced during myoblast differentiation, and to exert a myogenic function.[52]

Ubiquitously expressed miRNAs with myogenic functions. In addition to the muscle-restricted and muscle-enriched miRNAs described above, some ubiquitously expressed miRNAs also have myogenic functions, and their expression levels typically change during myoblast differentiation. ***MiR-214*** expression is de-repressed from the polycomb protein Ezh2 and stimulated by MyoD and myogenin during differentiation.[53] Similarly, ***miR-29b/c*** expression is suppressed in myoblasts by the repressive transcription factor YY1; during myoblast differentiation and muscle regeneration, release from YY1 and stimulation by SRF and MEF2 lead to the upregulation of miR-29 levels.[54] ***MiR-24*** transcription is also upregulated during myoblast differentiation, upon de-repression from TGFβ-Smad signaling.[55] De-repression from suppressive signals appears to be a common theme within this subset of myogenic miRNAs, the upregulation of which correlates with myogenic differentiation.

Several other miRNAs with myogenic functions are also upregulated during skeletal myogenesis, but the upstream regulators are yet to be identified. ***MiR-181*** is highly expressed in neurons and bone marrow but barely detectable in mature myofibers.[56,57] However, miR-181 levels are drastically increased during myoblast differentiation and muscle regeneration.[58] Other ubiquitously expressed myogenic miRNAs found to be upregulated during myogenic differentiation include ***miR-26a***,[38] ***miR-27b***,[59] ***miR-322/424*** and ***miR-503***.[60]

MiRNAs downregulated during myogenic differentiation. Compared to myogenic miRNAs that are upregulated, fewer miRNAs with demonstrated myogenic functions are downregulated during myogenic differentiation. ***MiR-221*** and ***miR-222,*** which share sequence similarity and are clustered on chromosome X, are found to be highly expressed in quail myoblasts, and downregulated during differentiation.[61] A similar expression pattern is found in mouse C2C12 myocytes, although the change is to a lesser degree.[61] It has been suggested that the expression of miR-221/222 is under the control of Ras-MAPK signaling,[61] consistent with a negative role that this pathway plays in myogenic differentiation.[62,63] MiR-222 expression has also been correlated with the presence of infiltrating inflammatory cells in injured muscles,[44] and it is highly upregulated in several muscular dystrophies.[64] Another miRNA downregulated during myoblast differentiation is ***miR-125b***,[65,66] a ubiquitously expressed and brain-enriched miRNA. Most recently we have identified a role for miR-125b as a suppressor of myogenic differentiation and muscle regeneration.[66] Expression of miR-125b at the transcriptional level is negatively controlled by mTOR signaling in an mTOR kinase-independent manner.[66] True to its emerging status of a master regulator, rapamycin-sensitive mTOR signaling regulates the biogenesis of two miRNAs in opposite directions—enhancing miR-1 levels[39] and suppressing miR-125b levels, through mTOR kinase-dependent (our unpublished observations) and -independent[66] pathways, respectively. Further delineation of the regulatory mechanism of miR-125b biogenesis will require identification of its cis regulatory elements, which is also the case for many of the other myogenically regulated miRNAs mentioned above.

MiRNA expression during skeletal myogenesis has been profiled extensively by numerous groups. Many miRNAs not mentioned above have been found to be differentially expressed during myoblast differentiation in vitro[38-40,47,52,67] and muscle development or disease in vivo.[44,47,64,68] Future characterization of those miRNAs will likely lead to the identification of novel myogenic regulators.

Gene Targets and Functions of Myogenic miRNAs

Manipulation of miRNA levels in vitro and in vivo, by genetic and biochemical methods, has become a standard approach to interrogating miRNA functions. Ultimately, revealing the gene targets of a miRNA is key to understanding its function. Identification of biologically important targets remains a major challenge in miRNA studies, as most metazoan miRNAs pair with their targets imperfectly. Pairing of miRNA "seed" region (nucleotide 2–8) to the 3'UTR of the target gene is believed to be one of the most important parameters that determine efficient miRNA targeting,[69-71] yet "seedless" targeting has also been well documented.[72,73] Although computational prediction has become a powerful tool in facilitating miRNA target identification,[18,74,75] the current algorithms remain far from accurate, with high false-positive and false-negative rates.[76] Experimental approaches to identifying miRNA targets continue to be developed and refined, but extracting biologically important information from typically very large data sets from such methods can be daunting. The reader is referred to Thomas et al.[77] for an excellent review on current approaches to miRNA target identification.

Despite the technical challenges, in recent years numerous biological targets for myogenic miRNAs have been revealed, representing a wide range of genes in the myogenic program, from transcription regulators, signaling molecules, to structural proteins. Here we survey the myogenic miRNAs for which gene targets have been identified or reasonably predicted (**Table 1**).

MiR-1 and miR-206. miR-1 and miR-206 differ by only four nucleotides outside the seed region, and they are believed to share gene targets although not all reported targets have been experimentally validated for both miRNAs. The gap junction protein connexin43 (Cx43) is a target of miR-1/miR-206,[78,79] the downregulation of which is necessary for myoblast fusion.[79] The receptor tyrosine kinase c-Met is also targeted by miR-1/miR-206, identified in a rhabdomyosarcoma cell line; miR-1/miR-206 could function as a potent tumor suppressor in c-Met-overexpressing tumors with aberrant proliferation and cell migration.[80,81] Another target of miR-1/miR-206 is histone deacetylase

4 (HDAC4),[40,82] a transcriptional repressor of muscle gene expression.[83] HDAC4 partly mediates the effects of miR-206 in promoting regeneration of neuromuscular synapses and delaying amyotrophic lateral sclerosis (ALS) in a mouse model.[82] Recently, we have found the myogenic fusion-promoting factor follistatin to be regulated by miR-1-controlled HDAC4, and uncovered an mTOR-MyoD-miR-1-HDAC4-follistatin pathway that regulates myocyte fusion in skeletal myogenesis.[39] Moreover, miR-1 and miR-206 have recently been reported to target Pax7 and, as a consequence, inhibit satellite cell proliferation and promote myogenic differentiation.[47,52,84] Interestingly, miR-1 and miR-206 levels undergo a temporary drop during the early phase of muscle regeneration in animals,[44,46,47,66] which correlates well with active satellite cell proliferation at that stage.[85]

Several more gene targets have been reported for miR-206, although it remains to be determined whether they are also shared by miR-1. Examining the effect of miR-206 overexpression on mRNA expression profiles in C2C12 myoblasts led to the identification of DNA polymerase alpha as a bona fide target of miR-206,[78] the downregulation of which may be involved in the suppression of DNA synthesis upon myogenic differentiation. Butyrate-induced transcript 1 (B-ind1), monocyte-to-macrophage differentiation-associated protein (Mmd), and Cx43 were also implicated as miR-206 targets in the same study.[78] As discussed earlier, Cx43 has been identified as a target for both miR-1 and miR-206 in another study.[79] More targets of miR-206 have been computationally predicted and experimentally confirmed, including follistatin-like 1 (Fstl1),[35] utrophin (Utrn),[35] and tissue inhibitor of metalloproteinase 3 (TIMP3).[86] Glucose-6-phosphate dehydrogenase (G6PD) has been reported to be a miR-1 target; in Duchenne muscular dystrophy the dystrophic phenotype is partially caused by HDAC2 suppression of miR-1 expression and subsequent G6PD over-production.[84]

MiR-133. Its genes clustering with those of miR-1 and miR-206, miR-133 targets SRF, thus enhancing myoblast proliferation and inhibiting myogenic differentiation.[40] Interestingly, miR-133 has also been shown to directly promote differentiation by targeting the alternative splicing factor neuronal polypyrimidine tract-binding protein (nPTB), the downregulation of which is necessary for the production of muscle-specific transcripts during myogenic differentiation.[87] MiR-133 is also reported to target uncoupling protein 2 (UCP2) known to regulate energy expenditure and thermogenesis in various organisms and to negatively impact myoblast differentiation.[88]

MiR-24. Found to be suppressed by TGFβ signaling, miR-24 promotes myoblast differentiation.[55] The targets of miR-24 in myogenesis have not been reported. Interestingly, in non-myogenic cells miR-24 has been found to target a cohort of cell cycle regulators, including Myc and E2F.[72] Hence, it is conceivable that miR-24 may target these same genes in myoblasts and support cell cycle exit necessary for myogenic differentiation.

MiR-26a. Upregulated during myoblast differentiation, miR-26a has been shown to promote differentiation.[38] The histone methyltransferase Ezh2, a polycomb group protein known to negatively regulate skeletal myogenesis,[89] is found to be a target of miR-26a.[38] Ezh2 binds to chromatin via its association with the transcription factor YY1, and subsequently silences muscle gene expression through its histone methyltransferase activity.[89] Hence, miR-26a suppression of Ezh2 may be necessary for myogenic differentiation. Dysregulated expression of miR-26a and Ezh2 has been found in rhabdomyosarcoma, implicating their involvement in the pathogenesis of this malignant tumor.[90]

MiR-27b. In a search for miRNAs that target Pax3, miR-27b emerged as a strong candidate predicted by multiple computational algorithms, and a direct targeting was confirmed experimentally.[59] Pax3 is required for muscle stem cell maintenance and migration, but its removal is necessary for myogenic differentiation.[91,92] MiR-27b promotes entry into the differentiation program both in vitro and in regenerating muscles by downregulating Pax3.[59]

MiR-29. MiR-29 targets the polycomb group protein associated transcription factor YY1.[54] YY1 recruits PcG proteins to suppress muscle gene expression, and is downregulated by NFκB transcriptionally[93] and by miR-29 post-transcriptionally[54] to allow myogenic differentiation. In addition, miR-29 targets collagen (COL1A1) and elastin (ELN) in the extracellular matrix in cardiac myocytes,[94] and the same targeting has been suggested to mediate the fibrosis phenotype in Duchenne muscular dystrophy due to suppression of miR-29.[84]

MiR-125b. As one of the few miRNAs downregulated during myogenic differentiation,[65,66] miR-125b is found to negatively regulate myoblast differentiation and muscle regeneration by targeting IGF-II,[66] a critical inducer of skeletal myogenesis.[95] As discussed earlier, mTOR signaling regulates miR-125b biogenesis in this context.[66] We have also reported that the transcription of IGF-II in differentiating myoblasts and regenerating muscles is regulated by mTOR.[96,97] Thus, mTOR controls myogenic IGF-II production at both transcriptional and post-transcriptional levels, highlighting the critical role of the mTOR-IGF-II axis in skeletal myogenesis. Other gene targets have been reported for miR-125b in non-myogenic cells, including Lin-28,[98] and p53.[99] Whether these genes are targeted by miR-125b in muscle and whether miR-125b targets IGF-II in non-muscle cells are yet to be determined.

MiR-181. The homeobox protein Hox-A11, reported to repress transcription of MyoD,[100] is found to be a target for miR-181.[58] The transient expression of miR-181 during myoblast differentiation and muscle regeneration, and the lack of its detection in adult muscles, suggest that this microRNA is involved in muscle formation but not maintenance.[58]

MiR-208b and miR-499. Produced from introns of two myosin genes—β-MHC and Myh7b, miR-208b and miR-499 share the same seed sequence and presumably the same set of targets. These two miRNAs function redundantly to target the transcriptional repressors of slow myofiber genes, including Sox6, Purβ, Sp3 and HP-1β, thus governing myofiber type switch.[49]

MiR-214. Like miR-26a, miR-214 also targets Ezh2,[53] a repressor of muscle gene expression. In addition, N-Ras has been found to be a target of miR-214 through transcriptional profiling of miR-214-overexpressing C2C12 myoblasts.[101] Since Ras promotes cell cycle entry and inhibits differentiation, miR-214 suppression of N-Ras facilitates cell cycle exit and subsequent

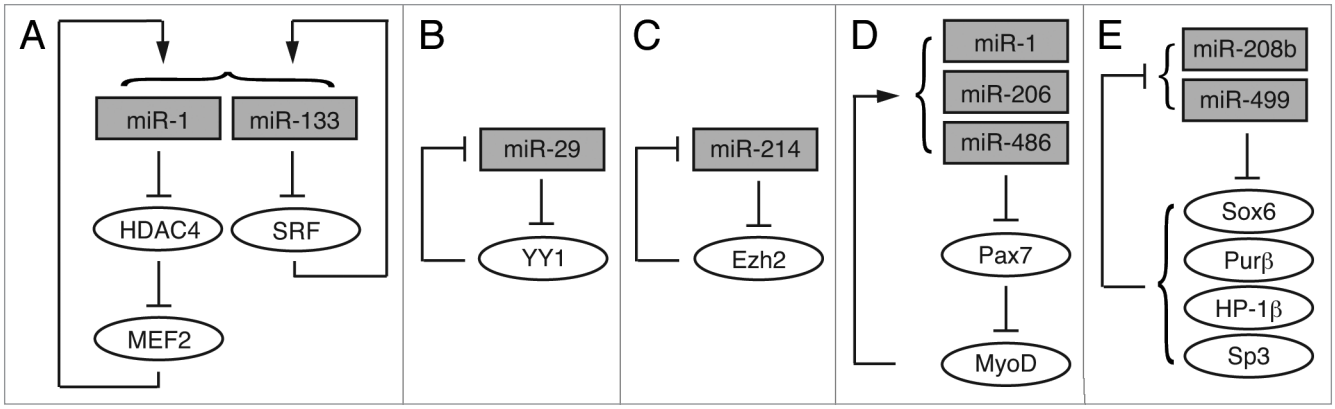

Figure 1. MiRNAs and their targets form feedback loops in the regulation of skeletal myogenesis. See text under "Regulatory feedbacks between miRNAs and their gene targets" for details.

myogenic differentiation.[101] In zebrafish, miR-214 is involved in muscle fate determination by modulating Hedgehog signaling.[102] It will be interesting to see whether this function is conserved in mammals.

MiR-221 and miR-222. MiR-221 and miR-222 target the cell cycle inhibitor $p27^{Kip}$ in cancer cells.[103,104] The same targeting has been implicated in myoblasts, and miR-221/miR-222 inhibits myogenic differentiation possibly through interfering with cell cycle withdrawal involving p27.[61] It has been suggested that p27 may also control myoblast differentiation through other cellular targets beyond cell cycle regulation.[105]

MiR-322/424 and miR-503. MiR-322/424 (miR-424 is the human ortholog of mouse miR-322) and miR-503 have very similar seed sequences, and their genes are clustered. These two miRNAs are reported to target Cdc25A,[60] a phosphatase responsible for removing inhibitory phosphorylation of Cdk2. Upregulation of miR-322/424 and miR-503 in differentiating C2C12 cells leads to cell cycle withdrawal through downregulation of Cdc25A, thus promoting myogenesis.[60]

MiR-486. Found to be enriched in cardiac and skeletal muscles, miR-486 was reported to target two negative regulators in the PI3K pathway—PTEN and FoxO1a—in rat cardiomyocytes.[51] Given the essential role of PI3K signaling in skeletal myogenesis,[106] it was a reasonable prediction that miR-486 would be required therein. Indeed, miR-486 has recently been shown to positively regulate myoblast differentiation.[52] However, in that study Pax7 is identified as the direct target of miR-486,[52] along with miR-206 (discussed earlier).

Emerging Themes of miRNA Regulation in Myogenesis

Some common themes have emerged from our current understanding of myogenic miRNA function and regulation. It is clear that these features are universal for miRNAs that function in all aspects of biology, rather than being unique to myogenesis.

Regulatory feedbacks between miRNAs and their gene targets. In several cases a miRNA is found to be under the regulation of its own target, forming a feedback loop. For instance, miR-1 targets HDAC4 and subsequently activates MEF2, which in turn stimulates miR-1 expression; at the same time, miR-133 targets SRF, a regulator of miR-133 transcription (**Fig. 1A**).[40] MiR-29 expression is suppressed by its target YY1 (**Fig. 1B**).[54] During myogenic differentiation, miR-29 is induced and it inhibits the expression of YY1, resulting in a robust upregulation of this myogenic miRNA.[54,107] In rhabdomyosarcoma cells and primary tumors where YY1 is elevated, miR-29 is silenced and differentiation is therefore impaired.[54,107] Similarly, miR-214 expression is suppressed in myoblasts by its target Ezh2. Upon differentiation Ezh2 is disengaged from the miR-214 promoter, which is then occupied and activated by MyoD and myogenin, leading to miR-214 expression. MiR-214 in turn downregulates Ezh2 expression, forming a feedback loop ensuring the progression of myogenic program (**Fig. 1C**).[53] In the case of miR-1, miR-206 and miR-486 (**Fig. 1D**), they all target Pax7,[47,52] which promotes the expression of Id2, an inhibitor of MyoD; the derepression of MyoD in turn activates the expression of these miRNAs, driving a MyoD-dependent differentiation state.[52] Another example is the intronic MyomiRs, miR-208b and miR-499 (**Fig. 1E**), that are expressed along with their host myosin genes and target the repressors of the transcription of those genes, thus effecting myofiber type specification and muscle performance.[49] This mode of feedback is likely to ensure robustness of responses that drive developmental switches such as myogenic differentiation.

Multiplicity of miRNA targeting. The combinatorial effects of co-functioning miRNAs and their multi-gene targeting are one of the defining features of miRNA regulation. MiRNAs having a common seed sequence may share gene targets, which can result in redundancy or cooperation of regulation. Myogenic miRNAs characterized so far that fall into that category include (pair-wise) miR-1/miR-206, miR-221/miR-222, miR-208/miR-499 and miR-322/424/miR-503. In addition, many myogenic miRNAs have closely related isoforms that most likely share gene targets and therefore function redundantly, although not all of them have been studied. The contribution of each miRNA isoform to a specific process will likely depend on the expression level of that isoform. For instance, of the two miR-125 isoforms, miR-125b appears to play a dominant role in negatively regulating skeletal

myogenesis while miR-125a levels in both myoblasts and myotubes are very low.[40,66,108] The functional redundancy of miRNAs extends beyond miRNA families, as it has become apparent that a gene can be simultaneously targeted by multiple miRNAs with unrelated sequences. Examples discussed in this review include Pax7 targeting by miR-1/miR-206 and miR-486, and Ezh2 targeting by miR-24 and miR-214. Conversely, a single miRNA often exerts its function by targeting multiple genes. MiR-1 and miR-206 illustrate this principle nicely, their reported targets including a DNA polymerase, a gap junction protein, a muscle structural protein, a transcriptional regulator, an oncogenic receptor tyrosine kinase and others. It is expected that many more cases of multi-targeting in both modes will be discovered, as myogenic miRNAs continue to be characterized.

Concluding Remarks

Over the last decade, our insights into miRNA regulation and function have grown exponentially, with paradigms being established, although working models are still constantly modified by the continuous output of a tremendous volume of data. The regulatory roles of miRNA have been established in almost every aspect of skeletal muscle development,[109] with a dozen or so myogenic miRNAs identified and their targets revealed (**Table 1**). However, this is unlikely to be a complete list. Future work, partly inspired by the sizable number of miRNAs found to be differentially expressed during skeletal myogenesis and powered by recent technological advances in biochemical purification of miRNA targets and deep sequencing,[77] will likely expand the family of myogenic miRNAs significantly. As our knowledge of individual myogenic miRNAs accumulates, it will be desirable and potentially feasible, to piece together a network of miRNAs and their targets and achieve an understanding of the circuitry at a systems biology level. Towards that goal, it will also be interesting to probe sufficiency of miRNA regulation—is there a subset of miRNAs that is sufficient for governing each key step of skeletal myogenesis? Adding individual or groups of miRNAs back into miRNA-depleted (e.g., Dgcr8 knockout) myocytes or muscles could help tackle this issue. Finally, as a new class of regulators of skeletal myogenesis, miRNAs hold the potential for the development of novel biomarkers and therapeutic strategies for muscular diseases.

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