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MicroRNAs Regulate and Provide Robustness to the Myogenic Transcriptional Network

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Abstract

The genetics of skeletal muscle lineage commitment are deceptively complicated. MyoD overexpression is sufficient to convert fibroblasts into skeletal muscle myotubes. In vivo, there are a number of different steps of differentiation that require a large network of transcription factors that control differentiation and homeostasis of skeletal muscle progenitors. Each transcription factor has been shown to have the ability to promote the next factor in the cascade, but the mechanisms regulating the transitions remain incomplete. Recently, microRNAs have been shown to be important for a large number of developmental and oncogenic processes. In this review, we will discuss recent advances in the understanding of how microRNA is critical for skeletal muscle development by interacting with protein-coding genes that had previously been shown to be important for myogenesis.

Introduction

Skeletal muscle precursor cells originate in the dermomyotome during early development. These progenitors are a population of proliferating and undifferentiated cells that express the paired-box transcription factors Pax3 and Pax7 [1]. The Pax genes are critical for commitment to the myogenic cell fate by inducing expression of the group of basic helix-loop-helix transcription factors referred to as the muscle regulatory factors (MRFs) in the dermomyotome (Fig. 1). The four MRFs are MyoD, Myf5, Myogenin and Mrf4. The MRFs were first identified for their ability to transdifferentiate fibroblasts into myotubes [2]. All of the MRFs heterodimerize with E proteins, and bind to the consensus DNA sequence CANNTG. However, mouse knockout studies show that despite some redundancy, each of the MRFs has distinct expression and function (reviewed in [3].) The muscle progenitors then migrate to the appropriate anatomical compartment and begin to form the skeletal muscle architecture. Skeletal muscle needs to retain the ability to regenerate in adulthood. Therefore adult skeletal muscle retains a reservoir of progenitor cells that are referred to as satellite cells due to their anatomical location outside of the fiber, but below the basement membrane [4]. These cells express Pax7, but usually not Pax3. The expression of Pax7 is crucial to proper satellite cell formation in development [5]. Satellite cells are normally quiescent but can divide either symmetrically to create two new progenitors, or asymmetrically to create one myoblast and one satellite cell [6]. The majority of satellite cells express the MRF Myf5, but about 10% do not, and muscle regeneration studies show

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the latter retain a greater capacity for satellite cell self-renewal. When these cells divide asymmetrically, the differentiating myoblast cell begins to express Myf5. This turns on several genes that both initiate differentiation and signal back to the non-differentiating cell via the Notch ligand Dll1 to maintain it in the stem cell mode. Among the myogenic genes turned on by Myf5 are the MRFs MyoD and Myogenin. Myogenin and Mef2c reciprocally induce each other [7] (Fig. 1). Together, these two transcription factors drive the maturation of myocytes into mature myotubes by regulating expression of myosin heavy chain proteins. The regulation of this transcriptional network involves a number of posttranscriptional pathways, including microRNAs.

MicroRNAs (miRNAs) are small non-coding regulatory RNAs with sizes of 17–25 nucleotides. miRNAs are coded either as unique genes, or found within an intron of a protein coding gene. Primary miRNAs are processed into hairpins by the Drosha/Dgcr8 complex [8]. The hairpin is then exported from the nucleus and processed by Dicer in the cytoplasm [9]. The mature miRNA then associates with the RISC complex and binds to the 3'UTR of target genes (reviewed in [10]). The mechanism of repression is predominately via a decrease in target mRNA stability [11]. The repression of a single gene by a single miRNA is not likely to be very strong, although this can be increased by having multiple target sites within the same 3'UTR. MiRNAs can also influence broader signal transduction pathways very strongly by targeting multiple players (reviewed in [12]). Skeletal-muscle specific knock-out of Dicer in mice results in gross abnormalities of fiber morphology and perinatal lethality [13]. There was no change in the levels of MRFs in Dicer knockout muscles when compared to control muscles. Therefore, it is probable that although MRFs themselves are not targeted by miRNA, proteins that control MRF activity will be. Accumulated evidence has demonstrated that steps in muscle transcription factor progression and induction of cell-cycle quiescence are regulated by miRNAs either directly or indirectly (summarized in Table 2).

Regulation of Pax3 and Pax7 by miRNA

Pax3 is critical for initiating the myogenic fate of cells in the dermomyotome [14]. It is also critical for the migration of progenitor cells to the developing limbs [15]. However, Pax3 must also be downregulated during terminal differentiation of skeletal myogenesis [16]. The 3'UTR of Pax3 contains a binding site for miR-27b, and transgenic overexpression of the microRNA decreases the expression of Pax3 in the myotome [17]. Inhibiting miR-27b in regenerating muscle led to an increase in Pax3 expression and fibers with a smaller diameter. Although correlative, this result suggests that regulation of Pax3 by miR-27b is important even in adult muscle regeneration. This conclusion could be strengthened by discovering the mechanism of miR-27b up-regulation during myogenesis. Work in cardiomyocytes has shown that miR-27b is repressed by the TGF-beta pathway [18], but this has not been demonstrated for skeletal muscle, nor is there any link between TGF-beta and Pax3 expression. In adult myoblasts, Pax3 is also down-regulated by the proteasome [16]. Expression of the MRF myogenin is mutually exclusive with expression of Pax3/7 by immunofluorescence [19], but the complete mechanism of feedback is unknown. The relative contribution of proteasomes and miRNA, and their coordination, to Pax3 downregulation is an outstanding question.

While Pax3 is important for embryonic development, Pax7 is essential for satellite cell development and maintenance in neonatal mice [20]. Activated satellite cells may transiently express both Pax7 and MRF genes, but ultimately the cell will either differentiate and decrease Pax7 or reduce MRF expression and remain as a satellite cell [19,21]. Pax7 and MRFs inhibit each other's activity (Fig. 1). Pax7 plays a direct role in repressing differentiation by directly promoting the transcription of the inhibitors Id2 and Id3 [22]. The

inhibitor of DNA binding (Id1–4) proteins are helix-loop-helix transcriptional repressors. Lacking a basic domain, they cannot bind to DNA, but instead bind to the E proteins that heterodimerize with the MRFs and titrate them away from MRFs and DNA. DNA binding by the MRFs is inhibited, inhibiting MRF function and stability [23]. The mechanism by which MRFs inhibit Pax7 involves microRNAs. Levels of mature miRNAs can be modulated by changes in transcription or processing [24]. Based on the hypothesis that MRFs may drive primary-microRNA transcription several groups manually searched for MRF-binding sequences proximal to the genomic loci encoding the most abundant miRNAs in skeletal muscle [25–28]. New high-throughput ChIP-seq data of MRF binding [29] allows for more efficient discovery of association between microRNAs and transcription factors. The miRNAs that contain at least one MyoD enriched region within 5 kb are summarized in Table 1. One of the first examples of transcriptional regulation was made between MyoD and miR-206 [26,27,30], explaining why miR-206 is almost nonexistent in tissues other than skeletal muscle [28]. Subsequently, miR-206 has been shown to directly target Pax7 [31,32], thereby explaining the mechanism by which MRFs downregulated Pax7. The robustness of this mechanism of regulation is two-fold. First miR-1 and -206 have very similar sequences and are likely to have largely overlapping mRNA target sets. Secondly, multiple miRNAs may be driven by the same transcription factor to repress the same target. In the case of Pax7, miR-486 is also transcriptionally activated by MyoD and targets the Pax7 3' UTR [32]. Overlapping mechanisms such as this may help explain why the phenotype of the miR-206 knockout was mild [33].

Regulation of MRF Activation

The Id proteins are not the only means of limiting the activity of MyoD. The MyoR protein contains a basic domain which permits it to directly compete for E-box binding. MyoR is upregulated in rhabdomyosarcoma, and prevents differentiation by repression of MyoD in those cells [34]. As in the case with Pax7, we hypothesized that there should be a feed-forward mechanism by which a MyoD transcribed gene represses MyoR. Indeed, miR-378 increases during myoblast differentiation and there are two MyoD binding peaks proximal to the miR-378 genomic locus [35]. MyoR contains a binding site for miR-378 in its 3' UTR. MyoR's ability to block MyoD mediated differentiation is negatively regulated by miR-378-binding elements in the MyoR 3' UTR. In contrast, inhibition of miR-378 did not completely block myoblast differentiation. A possible explanation is that a protein coding gene, Hes6, activated by MyoD can independently repress MyoR [36,37]. Therefore miRNAs may not only show redundancy with each other in repressing anti-differentiation factors, but also with proteins, thereby further increasing the robustness of the overall regulation of myogenic transcription factors.

Differentiation of skeletal muscle, as in many other tissues, involves changes in the cell cycle machinery. Prior to differentiation into myocytes, cells must leave the cell cycle and arrest in the G0 phase. Either overexpression of cyclin-dependent kinases (Cdk) [38], or knockout of their inhibitors p27 and p57 [39] is sufficient to prevent myogenesis. This is thought to occur because MyoD is normally phosphorylated and destabilized by the Cdk thereby blocking differentiation and promoting cell proliferation [40]. During differentiation, the expression of p27 and p57 increases, preventing the degradation of MyoD [41]. Upon stabilization, MyoD reinforces the cell-cycle exit by increasing expression of p21 [42], another Cdk inhibitor. However, unlike some of the loops discussed in this review, the induction of p27 and p57 is not dependent on MyoD and there are no known MyoD binding sites in their respective promoters. Both p27 and p57 are known targets of miR-221/222 [43,44], which decrease dramatically during skeletal muscle differentiation, thereby providing a microRNA-based mechanism for the increase of p27 and p57 [45]. While the effect of inhibiting MyoD is specific to skeletal muscle, the decrease in

miR-221/222 is seen in other differentiation pathways. Therefore the mechanism of downregulation of these microRNAs may not be a direct consequence of MyoD action, but is another example of how microRNAs may regulate skeletal muscle differentiation.

There are additional ways by which microRNAs induced during muscle differentiation inhibit the cell-cycle. The activity of Cdk is repressed by phosphorylation on threonine 14 and tyrosine 15, and the phosphatase Cdc25a increases Cdk kinase activity by removing these inhibitory phosphates. MiR-322 and miR-503 both target Cdc25a, thereby inhibiting Cdk [46]. MiR-322 and -503 both increase non-specifically in cells that are in the G0 phase of the cell cycle [47], indicating that their induction may be a general feature of post-mitotic differentiation. In addition, the MyoD induced microRNA miR-206 inhibits the DNA polymerase involved in DNA replication, the p180 subunit of DNA polymerase alpha, further inhibiting cell cycle progression and promoting differentiation [28].

Another signaling modality that interacts with MRF activity is the Insulin-like Growth Factor pathway (IGF). Although IGF signaling seems to have different effects during myoblast growth and differentiation [48], it is known to promote differentiation via the PI3K/Akt pathway [49,50]. This pathway directly regulates the processing of myogenic miRNAs via the RNA binding protein Khsrp [51]. The authors also showed that p-Akt increases the half-life of myogenin mRNA, but could not determine if this was a direct effect or a result of a decrease of a miRNA target. The IGF/PI3K/Akt pathway is regulated at a number of steps. IGFII is regulated by miR-125b, which decreases during differentiation, thereby increasing signaling [52]. The PI3K antagonist PTEN is regulated by miR-486, which is upregulated during differentiation, also increases signaling [53]. Uncontrolled activation of this pathway leads to hypertrophy (reviewed in [54]), so it would make sense to have a negative feedback loop. Both miR-1 [55] and -133 [56] serve this role by targeting the IGF receptor 1. This pathway has a large number of interactions and downstream effectors, so it appears to be fertile ground for further study.

Regulation of Mef2c and Muscle Fiber Differentiation

The MADS-box transcription factor Mef2c also has an important role in myogenic differentiation. A skeletal muscle specific knockout of Mef2c results in disorganized myofibers and perinatal lethality [57]. In differentiating myoblasts, Mef2c's activity is regulated by association with Hdac4 and Hdac5. These attach to the DNA-binding domain and prevent Mef2c's transcriptional activity [58]. MiR-1 forms a similar loop with Mef2c and Hdac4 as miR-206 does with MyoD and Pax7. The expression of miR-1 from both genomic loci requires Mef2c [59]. Expression of miR-1 then decreases levels of its target, Hdac4, thereby further promoting the transcription activity of Mef2c [60].

There is one interesting example where a microRNA is repressed to protect the activity of Mef2c. Both Mef2c and its cofactor Maml1 can be targeted by miR-135 and miR-133 respectively [61]. While the fold change of miR-135 is minimal, miR-133 is significantly increased during myoblast differentiation. Therefore a decrease of Maml1 during differentiation could be expected. However, the repression of Maml1 proteins is limited because of the simultaneous induction of a long non-coding RNA that serves as a decoy target for miR-133, preventing miR-133 from targeting Maml1 mRNA. Although this phenomenon has been shown in other contexts, it is still not clear exactly why the cell would express both a miRNA and a non-coding RNA to block its activity.

Skeletal muscle is not a completely homogenous organ. Fibers are commonly referred to as "fast" or "slow" and this is a reflection of which form of myosin heavy chain (Myh) is expressed. The type I slow fibers express the Myh7 and Myh7b genes. Each of these genes encodes a miRNA found within an intron, miR-208b and miR-499 respectively. These

miRNAs are homologous and mice with a knockout of one microRNA or the other display little phenotype [62]. The soleus (a slow muscle) of the double knockout mouse, however, shows a profound shift towards faster type IIa and IIx fibers from type I. A transgenic mouse with miR-499 expressed from an MCK promoter results in a uniformly type I soleus. In addition, the extensor digitorum longus features none of the very fast type IIb fibers. This phenomenon can be explained by the fact that both miR-208b and miR-499 repress the transcription factor Sox6, which promotes the expression of fast fiber Myh genes. Thus the induction of Myh7 and Myh7b in the slow fibers is accompanied by an induction of miR-208b and -499, which repress Sox6 and thus repress the fast fiber Myh genes. So in the same way that miRNAs are critical for earlier cell fate decisions of progenitors, they are also necessary for reinforcing decisions of which muscle fiber type to form.

Muscle-differentiation-induced microRNAs for disease therapy

There are many muscle wasting diseases and muscular dystrophies that could benefit from skeletal muscle tissue grafts. The process of integrating miRNA into more efficient differentiation is just beginning [63,64]. Similarly, miRNA is being used to increase the myogenic differentiation of embryonic stem cells [65]. Another translational possibility is with rhabdomyosarcoma (RMS), a cancer that shares many characteristics with skeletal muscle. Cell lines derived from this cancer can be differentiated into normal skeletal muscle with a MyoD-E12 tethered dimer, forcing the tumor to differentiate [34]. Forced expression of miR-206 can also attenuate tumor growth in mouse xenograft models with [66]. miR-29 [67] and miR-26a [68] also induce differentiation of RMS by targeting components of the polycomb group complex. Delivery of microRNAs has been proposed as a way to control the proliferation of cancers, including RMS. We suspect, however, that if microRNA is directly used for therapy, the tumor could escape by selecting for mutations in miRNA binding sites. However, knowing the network of genes regulated by miRNAs will allow us to find novel therapeutic targets against which we could develop chemical inhibitors that are likely to be more effective for therapy. In addition, critical microRNA targets often escape from microRNA control in during the pathogenesis of RMS, e.g. chromosomal translocations that separate the open reading frame of Pax3 or Pax7 from the microRNA-responsive 3'UTRs. MicroRNA delivery to such tumors is then expected to be futile for inhibiting Pax3 or Pax7. However, miRNAs may have some use for analysis of RMS aside from direct therapy. Expression of miR-206 has been found to be inversely correlated with the severity of the disease [69]. Patients with RMS also have higher levels of myogenic miRNAs in their plasma [70], which could be a minimally invasive way of monitoring response to therapy.

Conclusion

As the number of relevant miRNAs and targeted genes increases, the question arises as to how best to integrate all of this data. It is becoming less likely that individual miRNA-mRNA interactions are important for biological outputs or therapy; evolution would select against such a fragile network. As high-throughput technologies advance, the pace with which we can build networks of interactions should increase. Meta-analysis should allow us to find important nodes that would represent the most important therapeutic targets, as well as show the likely mechanisms of resistance. This should allow us to better design combination therapy for both developmental diseases and malignancies.

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Highlights

- MicroRNAs are important for proper embryonic development of skeletal muscle, as well as response to muscle injury
- The targets of microRNAs may be myogenic transcription factors, but also could be regulators of transcription factors
- Although a specific microRNA-mRNA interaction is rarely critical for myogenesis, it confers robustness to the overall network of regulation

Fig. 1.

A simplified flow chart of the order of expression and activation of transcription factors in secondary myogenesis. These interactions were all established prior to the discovery of the regulatory roles of miRNA.

Table 1

A list of miRNAs that have MyoD binding sites within 5 kb of their genomic locus. Also included are the relative changes in expression of the microRNA during the transition from myoblast to myotubes, and whether overexpression of that miRNA is sufficient to drive myoblast differentiation in normal growth media.

miRNA	Fold Change MT/MB	Differentiates MB	Reference
miR-133	+++	No	51
miR-1	+++	Yes	51
miR-206	+++	Yes	28
miR-26a	+++	Yes	56
miR-486	+++	Yes	32
miR-378	++	Yes	35
miR-762	++	Yes	N/A
miR-210	+	No	N/A
miR-22	+	No	N/A
miR-125b	–	No	57

Table 2

A summary of the mRNA-miRNA interactions discussed in this review and the nature of their effect.

Biological Effect	Target	miRNA
Target Pax Proteins	Pax3	miR-27b ¹⁷
	Pax7	miR-1/206/486 ^{31,32}
Activate MyoD	MyoR	miR-378 ³⁵
	Cdc25a	miR-322/503 ⁴⁶
Inhibit MyoD	p27/p57	miR-221/222 ^{43,44}
Activate Akt	Pten	miR-486 ⁵³
Repress Akt	Igf2	miR-125b ⁵²
	Igf1r	miR-1/133 ^{55,56}
Activate Mef2c	Hdac4	miR-1/206 ^{33,60}
Inhibit Mef2c	Maml1	miR-133 ⁶¹
	Mef2c	miR-135 ⁶¹
Bias Towards Slow Fibers	Sox6	miR-208b/499 ⁶²