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Role of microRNAs in skeletal muscle development and rhabdomyosarcoma (Review)

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Abstract. Skeletal muscle accounts for ~40% of total body mass. The principle functions of skeletal muscle include supporting the body structure, controlling motor movements and storing energy. Rhabdomyosarcoma (RMS) is a skeletal muscle-derived soft tissue tumor widely occurring in the pediatric population. In previous years, microRNAs (miRNAs) have been demonstrated to be important in skeletal muscle development, function and the pathogenesis of various diseases, including RMS. The present review provided an overview of current knowledge on the muscle-specific and ubiquitously-expressed miRNAs involved in skeletal muscle differentiation and their dysregulation in RMS. Additionally, the potential use and challenges of miRNAs as therapeutic targets in this soft-tissue sarcoma were examined and the future prospects for miRNAs in muscle biology and muscle disorders were discussed.

Contents

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1. Introduction

Muscle is an important part of the animal body and skeletal muscle constitutes ~40% of total body weight. The principal functions of skeletal muscle include maintaining body structure and posture, controlling motor movement and storing energy (1). Rhabdomyosarcoma (RMS) is a skeletal muscle-derived sarcoma occurring predominantly in children and young adults (2). There are two main subtypes of RMS: Embryonal RMS (eRMS) and alveolar RMS (aRMS). eRMS occurs more often in children <10 years old, whereas aRMS prototypically occurs in adolescents in 30% of RMS cases with a poorer prognosis and a higher rate of metastasis (3). In addition, aRMS exhibits typical chromosomal translocations between chromosomes 2 and 13 [t (2;13)(q35;q14)] or chromosomes 1 and 13 [t (1;13) (q36;q14)], which lead to the production of two fusion genes: paired box (PAX)3/forkhead box protein O1 (FOXO1) and PAX7/FOXO1, respectively (4). Furthermore, although RMS tumors commonly form from within skeletal muscle, they can also originate from non-muscle sites, including the skull base, genitourinary tract, biliary tree and salivary glands (5,6).

Previously, microRNAs (miRNAs), a novel class of small non-coding RNAs, have been demonstrated to act as key regulators of skeletal muscle cell fate determination and to be dysregulated in aRMS and eRMS (7). miRNAs are single-strand RNAs of ~22 nucleotides in length, which negatively regulate gene expression at the post-transcriptional level by complementary binding to the 3' untranslated regions of target genes and result in mRNA degradation or translation inhibition (8). To date, emerging evidence has demonstrated that miRNAs are critical in a considerable number of physiological and pathological processes, including proliferation, differentiation, chemoresistance and tumorigenesis (9-12). It was also reported that overexpression of selected 'tumor suppressor' miRNAs by gain-of function studies impaired the tumorigenic behavior of RMS cells (13). In addition, miRNA expression profiling has been demonstrated to be a promising approach to discriminate specific variants among RMS subtypes and further provide useful prognostic information concerning the alveolar and embryonal forms of RMS (14,15). These studies suggest that miRNA dysregulation may be involved in the pathogenesis of RMS.

The present review aimed to evaluate our current understanding of the regulation of miRNAs in skeletal muscle

development and their deregulation in RMS. Additionally, the possible therapeutic application and challenges of miRNAs in clinical practice were discussed.

2. Process of skeletal muscle development

The skeletal muscle system of vertebrates originates through a complex and multi-stage process termed myogenesis where numerous genes are co-operatively involved in the regulation of each stage (Fig. 1). This process begins in the somites of the embryo, which differentiate into dermomyotome-containing myogenic precursors at the first stage. Following commitment to a myogenic cell lineage, the myogenic precursors proliferate and differentiate into myoblasts, followed by differentiation into myotubes and finally differentiate into myofibers (16).

The regulatory network leading to the process of muscle development has been ascribed to a specific class of transcription factors termed myogenic regulatory factors (MRFs) (17). The expression of MRFs is limited to the muscle lineage and results in the activation of a cascade of events leading to the formation of mature muscle fibers.

The upstream regulators of early MRFs are paired-domain- and homeobox-containing proteins, including Pax3 and Pax7, which are active in embryogenesis. As myoblasts migrate, myogenin (MyoG) and MRF4 are expressed and trigger myoblasts to differentiate into myotubes. Following the terminal differentiation of myotubes, they act in concert with other factors, including myocyte enhancer factor 2 (MEF2) and serum response factor (SRF) to activate genes responsible for muscle fiber architecture and functionality (18). Besides these intrinsic signaling pathways, the differentiating muscle cells are regulated by external stimuli, including transforming growth factor (TGF)-β or Wnt signaling (19). In addition, the majority of the aforementioned processes can also be modulated at the post-transcriptional level by miRNAs, which are demonstrated to be irreplaceable in skeletal muscle development.

3. Expression patterns of miRNAs in skeletal muscle development

During skeletal muscle development, certain miRNAs are specifically enriched in skeletal muscle cells and others are differentially expressed in the development process. The temporal or tissue specific expression patterns of miRNAs have been determined by miRNA array or high-throughput sequencing approaches in previous years(20). In one study, 77 miRNAs were found to be upregulated and 68 miRNAs were downregulated by microarray in C2C12 myoblast cells, which were induced to differentiate in horse serum (20). Among the 77 upregulated miRNAs, miR-133a-1, miR-133a-2, miR-133b and miR-206 were the most significantly upregulated. Their critical role in skeletal muscle differentiation was also confirmed by other studies (21-24). Several other miRNAs, including miR-9-2, miR-122a, miR-703 and miR-805, were most significantly downregulated, however, few of them were found to be involved in the differentiation process. Certain miRNAs, including miR-699a, were downregulated during skeletal muscle differentiation. These observations demonstrated that miRNAs were differentially expressed in the process of skeletal muscle development.

4. Muscle-specific miRNAs in myogenesis

Numerous miRNAs can be highly and specifically enriched in certain tissues. The miRNAs that are specifically expressed in skeletal muscle are referred to as myomiRs, which include the miR-1/206 cluster (21,25). A complete list of myomiRs is provided in Table I and their function is further described in corresponding paragraphs. The miR-1/206 cluster is composed of six distinct miRNAs located on three separate chromosomes in three bicistronic transcripts. miR-1-2 and miR-133a-1 are located on chromosome 18, miR-1-1 and miR-133a-2 are located on chromosome 20 and miR-133b along with miR-206 are located on chromosome 6 (Fig. 2). In terms of architecture, miR-1-1 and miR-1-2 are identical and differ from miR-206 by four nucleotides. miR-133a-1 and miR-133a-2 are identical and differ from miR-133b by one nucleotide. During myogenesis, the myogenic transcription factors MyoD, MEF2 and SRF directly regulate the expression of miR-1 and miR-133a in skeletal muscle whereas the expression of miR-206 is controlled by MyoD and MyoG (26,27).

The function of the muscle-specific miRNAs in myogenesis has been examined in detail. It was reported that miR-1 and miR-133a regulate skeletal muscle cell proliferation and differentiation by targeting histone deacetylase 4 (HDAC4) and SRF, respectively, thus establishing a negative-feedback loop for myocyte differentiation (21). Furthermore, the injection of miR-1 into embryonic cardiomyocytes of mice led to decreased proliferation of cardiomyocytes, which was ascribed to the decreased expression of Hand2, a transcription factor that promotes cardiomyocyte proliferation (26). Consistently, a significant increase in Hand2 expression and proliferating cardiomyocytes was observed in an miR-1-2 deficient mouse model (28).

By contrast, miR-133a promotes myoblast proliferation partly by repressing the expression of SRF, a critical regulator of muscle cell differentiation (21). The genetic interaction between miR-133a and SRF results in the upregulation of miR-133a by SRF leading to the further repression of SRF, thereby constituting a negative feedback loop. Paradoxically, miR-133a and miR-1 exhibit opposing effects on skeletal muscle development although they derive from the same miRNA polycistronic transcript. The primary function of miR-133a is to promote proliferation and inhibit differentiation, while the function of miR-1 is to induce the differentiation of mesodermal progenitors to the muscle lineages. It has been demonstrated that miR-1 and miR-133 have a specific role in muscle cell proliferation and differentiation in an antagonistic manner, with the balance being altered one way or the other by additional modulators of gene expression (29).

Similar to miR-1 in skeletal muscle, miR-206 has been demonstrated to promote myoblast differentiation by repressing the expression of connexin 43 (Cx43), thereby decreasing the electrical coupling between myofibers via gap junctions, which inhibits the terminal differentiation of skeletal muscle cells (30). In addition, miR-206 was reported to repress the expression of the p180 subunit of DNA polymerase α1 (22), Pax7 (31), follistatin-like 1 (32) or utrophin (32), thereby suppressing muscle cell proliferation through inhibiting DNA synthesis.

Table I. Muscle-specific miRNAs involved in myogenesis and RMS.

miRNA	Target gene	Function	Reference
miR-1	HDAC4	Promotes myoblast differentiation	20
	Hand2	Inhibits cardiomyocyte proliferation	25,27
	PAX3	Inhibits RMS cell proliferation	41
	PAX7	Promotes muscle cell differentiation	29
	CCND2	Inhibits RMS cell proliferation	41
	cMet	Inhibits RMS development	40
miR-133	SRF	Promotes myoblast proliferation	20
miR-206	PAX7	Inhibits muscle cell proliferation	29
	PAX3	Inhibits RMS cell proliferation	41
	CCND2	Inhibits RMS cell proliferation	41
	cMet	Inhibits RMS development	40
	Cx43	Promotes myoblast differentiation	28
	Polα1	Inhibits muscle cell proliferation	21
	Fstl1	Inhibits muscle cell proliferation	30
	Utrn	Inhibits muscle cell proliferation	30

miRNA, microRNA; RMS, rhabdomyosarcoma; HDAC4, histone deacetylase 4; PAX, paired box; SRF, serum response factor; Cx43, connexin 43; Polα, DNA polymerase α; Fstl1, follistatin-like 1; Utrn, utrophin.

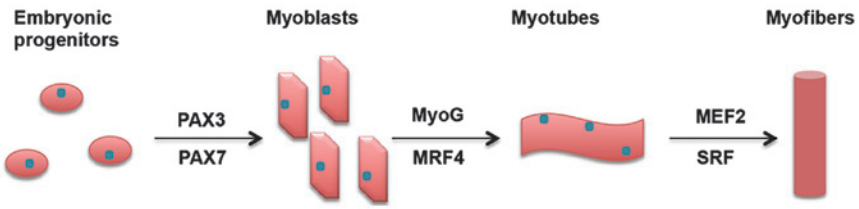

Figure 1. Schematic illustration of skeletal muscle development. During myogenesis, PAX3 and PAX7 are initially activated in embryonic progenitors. With the continuous development of embryos, certain embryonic progenitor cells differentiate into muscle precursor cells termed myoblasts. As myoblasts migrate, MyoG and MRF4 are expressed, which stimulate myoblasts to differentiate into myotubes. They then act with MEF2 and SRF to activate genes responsible for muscle fiber (myofibers) architecture and functionality. PAX, paired box; MyoG, myogenin; MRF4, myogenic regulatory factor 4; MEF2, myocyte enhancer factor-2; SRF, serum response factor.

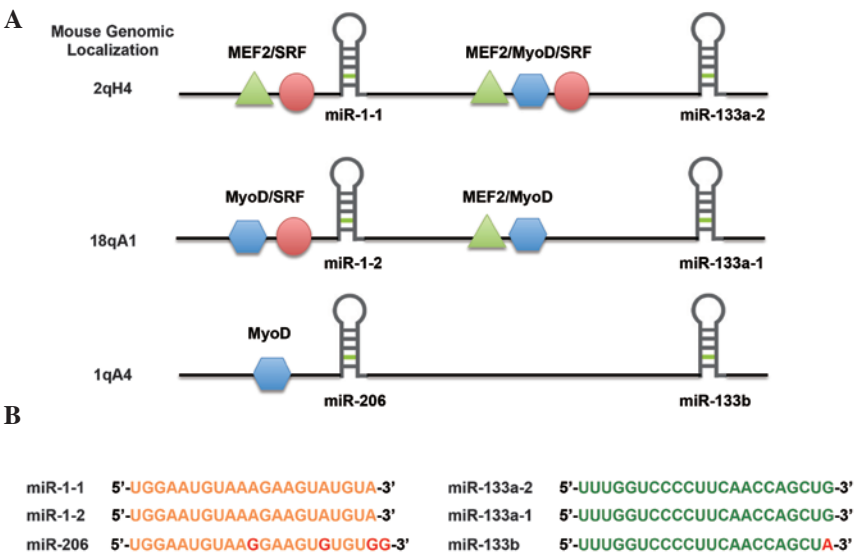

Figure 2. Schematic overview of three myomiR clusters. (A) Genomic locations of the three bicistronic myomiR clusters, including miR-1-1/miR-133a-2, miR-1-2/miR-133a-1 and miR-206/miR-133b on mouse chromosomes. The myogenic regulatory factors SRF, MEF2 and MyoD are also indicated. (B) Comparison of the sequences of three bicistronic myomiR clusters (5'-3'). Red indicates the different nucleotides among the miRNA families. miR, microRNA; MEF2, myocyte enhancer factor-2; SRF, serum response factor.

Table II. Non-muscle-specific miRNAs involved in myogenesis and RMS.

miRNA	Target gene	Function	Reference
miR-27b	PAX3	Promotes myoblast differentiation	31
miR-26a	Ezh2	Promotes myoblast differentiation	32
miR-214	Ezh2	Promotes myoblast differentiation	33,34
miR-181	HOX11	Promotes muscle cell differentiation	35
miR-669a	MyoD	Inhibits skeletal muscle differentiation	36
miR-669q			
miR-29	YY1	Promotes myoblast differentiation	42
	PAX3	Inhibits RMS cell proliferation	41
	CCND2	Inhibits RMS cell proliferation	41
miR-183	PTEN	Promotes RMS cell migration	43
	EGR1	Promotes RMS cell migration	43
miR-203	P63	Inhibits RMS cell proliferation	44
miR-9[a]	E-cadherin	Inhibits RMS cell migration	45
miR-450b	TGF-β1	Inhibits RMS development	46

miRNA, microRNA; RMS, rhabdomyosarcoma; PAX, paired box; Ezh2, enhancer of zeste 2; HOX11, homeobox-protein A11; YY1, Yin Yang 1; PTEN, phosphatase and tensin homolog; EGR1, early growth response 1; TGF-β1, transforming growth factor-β1.

5. Non-muscle-specific miRNAs in myogenesis

In addition to muscle-specific miRNAs, numerous non-muscle-specific miRNAs, referred to as non-myomiRs, are also important in the regulation of myogenesis (Table II). It has been demonstrated that these non-myomiRs regulate muscle proliferation and differentiation through the repression of target genes through multiple processes (33-39). At the onset of myogenesis, miR-27b induced improper migration and early differentiation of myoblasts by targeting the Pax3 protein (33). miR-26a (34) and miR-214 (35,36) also promote myogenesis by targeting enhancer of zeste 2, another known inhibitor of myogenesis. It is noted that the timing of expression of miR-26a and miR-214 differs during myogenesis. Once muscle differentiation begins, miR-214 is upregulated via MyoD/MyoG, which promote $P21^{Cip1}$ and myogenin expression, while miR-26a increases gradually during the course of myogenesis.

Inhibition of homeobox-protein A11 (HOX11) by miR-181 is another step in muscle differentiation (37). The low expression of HOX11 leads to increases in MyoD, a target of HOX11, and proper differentiation in muscle cells. It is consistent with the finding that miR-181 is upregulated during muscle development whereas it is downregulated in adult skeletal muscle (38). By contrast, miR-669a and miR-669q are expressed in the heart muscle to prevent skeletal muscle differentiation from the beginning through inhibiting MyoD and its targets, thus ensuring that skeletal muscle myogenesis occurs in the correct locations (39).

Taken together, these observations are consistent with differential expression profiles of miRNAs between myogenesis and the adult skeletal muscle. The differentially expressed miRNAs provide a molecular basis for proper regulation of muscle development, which highlights the complexity of miRNA function.

6. Muscle-specific miRNAs in rhabdomyosarcoma

RMS is predominantly a pediatric sarcoma that resembles developing skeletal muscle and accounts for >50% of soft tissues sarcomas in children (40). Evidence has demonstrated that myomiRs were significantly downregulated in RMS, indicating a critical role in the terminal differentiated phenotype of RMS cells (41). In particular, Rao *et al* (29) reported that overexpression of miR-1 in the RMS cell line, RD, results in muscle gene expression and cell cycle arrest, whereas miR-133a decreases the expression of muscle markers. This is consistent with the distinct roles of miR-1 and miR-133a in normal muscle differentiation. However, miRNAs suppress cell growth in the RMS cell line, indicating that cell context is important to the fate of miRNA regulation.

A similar growth inhibitory effect has also been confirmed by forced expression of either miR-1 or miR-206 in the RMS cell line *in vitro* and *in vivo* (42,43). The induction of miR-1/206 precursor led to decreased myogenic differentiation in cell migration and inhibition of tumorigenic potential. Furthermore, the results of mRNA profiling prior to and following miR-206 transfection in RD18 cells revealed that >700 genes were modulated, including c-Met (43). The downregulation of c-Met by miR-1/206 led to a significant inhibition of RMS development, suggesting that the targeting of c-Met is one of the underlying mechanisms responsible for RMS development.

The anti-tumor capacity of the ectopic expression of the miR-1/206 cluster in RMS was further verified by the observation that these miRNAs directly regulate the expression of CCND2, a cell cycle gene (44). Overexpression of miR-1/206 demonstrated a strong promyogenic effect in RMS cells and downregulated the protein and transcript levels of CCND2. Additionally, miR-1/206 significantly downregulated PAX3 protein expression in the eRMS cell line, JR1, however,

demonstrated no effect on the protein levels of PAX3 in an aRMS cell line, Rh30 (44). This finding highlights, once more, the importance of cell context in determining the response to miRNA modulation.

7. Non-muscle-specific miRNAs in rhabdomyosarcoma

In addition to myomiRs, numerous non-myomiRs are implicated in the regulation of RMS development. The deregulation of miR-29 was reported in a small cohort of aRMS in which nuclear factor-κB activation led to overexpression of Yin Yang 1, resulting in sustained downregulation of miR-29b2/miR-29c and inhibition of myogenesis (45). In addition, decreased expression of miR-29, as well as miR-1/206, stabilized the RMS phenotype by targeting PAX3 and CCND2 (44). These findings reiterate that the RMS state is maintained by the deregulation of multiple miRNAs and their target genes, supporting a tumor suppressor role for these miRNAs.

Another miRNA linked to RMS is miR-183, which acts as an onco-miR in several types of cancer, including RMS, synovial sarcoma and colon cancer (46). Knocking down of miR-183 by anti-miR-183 treatment in tumor cells reduced cell migration *in vitro* and stimulated the expression of the tumor suppressor gene phosphatase and tensin homolog (PTEN), which in turn, promoted early growth response 1 (EGR1) expression, thus reinforcing the repression of cell migration (46). These results demonstrated that miR-183 has an oncogenic role through targeting two tumor suppressor genes, EGR1 and PTEN, and the deregulation of the fundamental miRNA regulatory network may be central to the development of several other tumor types.

Additionally, certain other non-myomiRs were described in the context of cell differentiation, migration or metastasis in RMS. Re-expression of miR-203 in RMS cells inhibited their proliferation and migration and promoted terminal myogenic differentiation by directly targeting p63 (47). miR-9^{a} is another miRNA capable of inhibiting cell migration, which was found to directly target E-cadherin and was expressed in higher levels in aRMS than in eRMS, correlating with their metastatic potentials (48). In addition, in the two cultured cells and tumor implants, the growth of RMS was significantly arrested by miR-450b-5p, which was strictly regulated by TGF-β1 (49).

Taken together, these data demonstrate the critical role of miRNAs in modulating target genes involved in one or more cellular function/process and the complexity of miRNA regulation and function in RMS development. In addition, these studies have revealed that small gene expression alterations, even if only occurring in one miRNA, may be affect the balance between pathological and physiological cell fate programs.

8. miRNAs as novel therapeutic targets in rhabdomyosarcoma

The widespread and crucial roles of miRNAs in RMS development and progression raise interesting prospects for exploiting miRNAs as novel therapeutic targets in RMS.

In this regard, various approaches that upregulate or downregulate miRNAs have been employed to target miRNAs in RMS, and demonstrate significant efficacy in the treatment of RMS development following intravenous delivery *in vivo* (50). In particular, two pre-clinical studies demonstrated that ectopic expression of miR-206 by lentiviral vectors leads to cell cycle arrest and myogenic differentiation of RMS cells, preventing xenograft growth *in vivo* by inhibiting the expression of oncogenic c-Met (42,43). In addition, knockdown of miR-183, an onco-miR in several types of cancer, by antisense-based miRNA antagonists led to significant decreases in tumor migration through directly promoting the expression of EGR1, a regulator of cell migration (46).

Although the upregulation or downregulation of selected miRNAs is a possible strategy for targeted therapy in RMS, it must be noted that there remain several challenges regarding miRNA-based therapy. Viral vectors, though efficient in the overexpression of miRNA genes, are limited in their clinical application by immunogenicity and non-specificity. Non-viral cationic liposomes are attractive for mediating miRNA transfer, however, their low efficiency in cell transfection also limit their development. Certain types of nanoparticles have been proposed to efficiently deliver miRNAs or anti-miRNAs to target tumor sites (51), implying that they are alternative tools for introducing miRNAs for the treatment of RMS. However, additional preclinical data are required to demonstrate their suitability for the clinic and efficacy in the application of miRNA therapy. Their relevance and mode of action require further investigation in genetic models of RMS that more accurately recapitulate the onset and progression of aRMS and eRMS tumors.

9. Conclusion

miRNAs have emerged as critical regulators in skeletal muscle development, regeneration and function. They are also found to be dysregulated in skeletal muscle-associated diseases, including RMS. Thus, miRNAs are promising biomarkers and candidates for potential therapeutic intervention, and provide an avenue to further dissect the mechanisms that may contribute to genetic and acquired muscle disorders or other associated diseases. Future studies are required to focus on the identification of miRNAs involved in skeletal muscle development and on advancing novel therapies that are able to modulate miRNA activity to treat muscle-associated diseases.

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