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MicroRNAs in the regeneration of skeletal muscle

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1. ABSTRACT

MicroRNAs (miRNAs) have emerged as critical regulators of numerous biological processes by modulating gene expression at the post-transcriptional level. The discovery of miRNAs as new and important regulators of gene expression is expected to broaden our biological understanding of the regulatory mechanism in muscle by adding another dimension of regulation to the diversity and complexity of gene-regulatory networks. Exercise-induced skeletal muscle injury and repair have always been among the highlights of research in sports medicine. However, many mechanism problems that occur during regeneration and repair in the aftermath of skeletal muscle injury remain unsolved. It has become increasingly clear that the regeneration of skeletal muscle development involve regulation by miRNAs. In the last few years the field has seen a rapid expansion of our knowledge of miRNAs in the regeneration of skeletal muscle. This study reviews the miRNAs related with regeneration of skeletal muscle, and discuss the regulation of their expression in muscles, and emerging themes of miRNA regulation.

2. INTRODUCTION

MicroRNAs (miRNAs) are a class of conserved noncoding RNAs aobut 22 nucleotides, which regulate their target gene expression posttranscriptionally (1). In 1993, Lee *et al.* (2) discovered the first non-coded small molecule RNA lin-4. In 2000, Reinhart (3, 4) found let-7 in Caenorhabditis elegans and gave the name “microRNA” to the non-coding RNA of this endogenous, single-chain small molecule that is able to regulate the developmental process. Among the hundreds of miRNA discovered so far, only a few have been found functional, with their target genes experimentally proven (5). Similar to other genes, miRNA also come from genome coding and transcription. miRNA is transcribed into primary miRNA 300-1000 nucleotides long. Ribonucleases, such as Drosha, cut the nucleus, which transforms it into precursor miRNA between 70-100 nucleotides long. Afterward, it is transferred to the cytoplasm and generates a mature miRNA under the effect of Dicer, another ribonuclease. The mature miRNA is between 15-20 nucleotides long and its sequence is highly conserved among species (6, 7). However, its degradation

mechanism is still unclear. By combining it into the 3'-end non-coding zone of the corresponding target mRNA, miRNAs act as potent negative regulators of protein translation by disrupting the stability of mRNA, which realizes the post-transcription regulation of genetic expression and plays an important role in physiologic processes, such as the regulation of the normal development of organisms (8).

Exercise-induced skeletal muscle injury and repair have always been among the highlights of research in sports medicine. Skeletal muscle has a remarkable capacity to respond to pathological stress and regenerate upon injury. Skeletal muscle consists of postmitotic, multinucleated myofibers and mononuclear cells, including muscle progenitor cells (satellite cells) and other less well-characterized interstitial cells such as muscle side population (SP) cells. Muscle regeneration is mainly dependent on satellite cells, which in response to injury undergo proliferation, differentiation, and ultimately, fusion with new or existing myofibers to repair the muscle. However, many mechanism problems that occur during regeneration and repair in the aftermath of skeletal muscle injury remain unsolved. Here we will review the miRNAs related with regeneration of skeletal muscle, and discuss the regulation of their expression in muscles, and emerging themes of miRNA regulation.

3. SKELETAL MUSCLE-SPECIFIC MIR-206 IN THE REGENERATION OF SKELETAL MUSCLE

MiR-206 was initially cloned by Lagos-Quintana and colleagues (9) from human and murine muscular tissues in 2003. It has a length of 22 nucleotides and encodes genes located in chromosome No. 6. The expression of miR-206 increased in the differentiation and maturation of skeletal muscular cells and displayed time-space specificity to a certain extent (10). The skeletal musclespecific expression of miR-206 was first clearly demonstrated by microarray analysis and later confirmed by Northern blot (11, 12). Now, miR-206 has been accpeted as the skeletal muscle-specific muscle miR (13).

3.1. Expression of miR-206 in skeletal muscle

A study with MDX mice and Duchenne muscular dystrophy patients (DMD signature) aiming to identify miRNAs involved in the pathological pathways activated in skeletal muscle damage and regeneration by both dystrophin absence and acute ischemia found that miR-206 was up-regulated following myoblast differentiation *in vitro*. This finding show an important role of miRNAs in physiopathological pathways regulating muscle response to damage and regeneration (14). *In vitro* study later found an increased expression of miR-206 in C2C12 cells differentiation and cardiotoxin (CTX)-induced regeneration, in which differentiated myotubes or regenerating fibers showed abundant expression of miR-206 (15). These observations indicated that newly formed myotubes showed markedly increased expression of miR-206, which might reflect active regeneration and efficient maturation of skeletal muscle fibers (15). *In vivo* study demonstrated that miRNA-206 was overexpressed in the diaphragm but not the hindlimb muscle of dystrophin-deficient (mdx) mouse (16). John and colleagues found that in the soleus and plantaris, expression of the mature miR-206, was decreased by approximately 25%, whereas in the diaphragm, miR-206 expression increased by 4.5-fold relative to control. The increased expression of miR-206 in the mdx diaphragm was paralleled by a 4.4-fold increase in primary miRNA-206 (pri-miRNA-206) transcript level (16). Both *in vitro* and *in vivo* studies indicate an important role of skeletal muscle-specific miR-206 in the regeneration of skeletal muscle.

3.2. MicroRNA-206 in the regulation of satellite cells

In vitro and *in vivo* studies have begun to elucidate the role of miR-206 during regeneration of skeletal muscle. The direct target genes regulated by miR-206 include connexin 43 (CX43), histone deacetylase 4 (HDAC4), and so on. Mutual negative feedback regulation exists between TGF-beta and miR-206. In the myogenic process of skeletal muscle, CX43 and HDAC4 inhibit the expression of specific genes, and miR-206 induces the expression of specific muscular genes to inhibit CX43 and HDAC4. This process involves the upregulation of the expression of various growth factors (e.g., IGF-I); and TGF-beta inhibits myogenic differentiation by suppressing miR-206 expression, which increases HDAC4 upregulation and subsequently inhibits the expression of specific muscular genes (17, 18,19).

In the regeneration of skeletal muscle, miR-206 participates to regulate the activation of satellite cells, which would be an significant mechanism of miR-206 involving into regeneration of skeletal muscle. Satellite cells are stem cells of the adult muscle, which display stem cell properties such as self-renew and giving rise to progeny that can undergo myogenic differentiation *in vitro* and *in vivo* (20, 21). When skeletal muscle is injured, quiescent satellite cells surrounding the damaged myofibers are activated and proliferate forming huge numbers of myoblast progeny cells which then differentiate and are either incorporated into existing damaged myofibers or undergo fusion with each other forming new myofibers (22). A subset of these expanded progeny cells do not undergo differentiation and revert back to the quiescent state thus re-establishing the stem cell pool on the newly formed myofibers (23). These characteristics enable satellite cells to regenerate damaged muscle as well as permitting growth. Myogenic differentiation in adult muscle is normally suppressed and can be activated by myogenic cues in a subset of activated satellite cells. However, the switch mechanism that turns myogenesis on and off is not defined. Liu and colleagues demonstrate that tissue inhibitor of metalloproteinase 3 (TIMP3), the endogenous inhibitor of TNF-alpha-converting enzyme (TACE), acts as an on-off switch for myogenic differentiation by regulating autocrine TNF-alpha release (24). More importantly, downregulation of TIMP3 is mediated by the myogenesis-promoting miR-206 in C2C12 myoblasts (24). More recently, other team revealed an essential role for miR-206 in satellite cell differentiation during skeletal muscle regeneration (25). They showed that miR-206 promotes skeletal muscle regeneration in response

to injury. Genetic deletion of miR-206 in mice substantially delayed regeneration induced by cardiotoxin injury. And miR-206 acts to promote satellite cell differentiation and fusion into muscle fibers through suppressing a collection of negative regulators of myogenesis (25). MiR-206 also was demonstrated to involve into regulating MyoD and Pax genes, which will be reviewed later.

3.3. MiR-206 related signalings in the regeneration of skeletal muscle

MiR-206 participates regeneration of skeletal muscle by regulating some signaling pathways. In a study with mouse model of amyotrophic lateral sclerosis (ALS), miR-206 was found to be required for efficient regeneration of neuromuscular synapses after acute nerve injury (26). miR-206 mediates these effects at least in part through fibroblast growth factor signaling pathways (26). In addition, miR-206 might play a role in regulating retrograde signaling of brain-derived neurotrophic factor (BDNF) at the neuromuscular junction (27). BDNF is required for efficient skeletal-muscle regeneration and perturbing its expression causes abnormalities in the proliferation and differentiation of skeletal muscle cells. Transfection of a miR-206 precursor decreased activity of reporters representative of the BDNF-L 3' untranslated region, but not BDNF-S 3' untranslated region, and repressed endogenous BDNF mRNA levels. This suppression was found to be dependent on the presence of multiple miR-206 target sites in the BDNF-L 3' untranslated region. Conversely, suppression of miR-206 levels resulted in de-repression of BDNF 3' untranslated region reporter activity and increased endogenous BDNF-L mRNA levels. These results suggested that miR-206 might regulate retrograde signaling of BDNF at the neuromuscular junction (27).

4. OTHER MICRORNAS

In addition to miR-206, some other miRNAs have been found to participate the regeneration of skeletal muscle. MiR-181, which is strongly upregulated during differentiation, was found to participate in establishing the muscle phenotype (28). Moreover, miR-181 could downregulates the homeobox protein Hox-A11, which is a repressor of the differentiation process, thus establishing a functional link between miR-181 and the complex process of mammalian skeletal-muscle differentiation (28). In the study carried out by John and colleagues, expression of the mature miR-133a was decreased by approximately 25% in the soleus and plantaris of the mdx mouse (16). Regeneration miRNAs, including miR-31, miR-34c, miR-335, miR-449, and miR-494, which were induced in MDX mice and in Duchenne muscular dystrophy patients, but also in newborn mice and in newly formed myofibers during postischemic regeneration. Notably, miR-34c and miR-335 were up-regulated following myoblast differentiation *in vitro* (14). MiR-31 expression was also found to be related with nutrient-related hormones in the loss of muscle mass and myofiber size with aging in mice, as well as miR-223 (29). Both miR-31 and miR-223 have been known to be elevated during muscle regeneration and repair.

The combination of double-stranded (ds) miR-1 and 133, as well as miR-206, can promote myotube differentiation.Nakasa and colleagues found that a local injection of miR-1 and miR-133 can accelerate muscle regeneration in a rat skeletal muscle injury model. After the laceration of the rat tibialis anterior muscle, miR-1 and 133, as well as miR-206, mixture mediated atelocollagen was injected into the injured site. The control group was injected with single-stranded (si) RNA. At 1 week after injury, an injection of dsmiRNAs could enhance muscle regeneration morphologically and physiologically, and prevent fibrosis effectively compared to the control siRNA (30).

A study about aging with loss of skeletal muscle and miRNAs expression in human skeletal muscle by a microarray and bioinformatics analysis suggested that Let-7 expression may be an indicator of impaired cell cycle function possibly contributing to reduced muscle cell renewal and regeneration in older human muscle (31). In that study, Let-7 family members Let-7b and Let-7e were significantly elevated and further validated in older subjects. The Let-7 family members were associated with molecular networks involved in cell cycle control such as cellular proliferation and differentiation, suggesting that aging is characterized by a higher expression of Let-7 family members that may downregulate genes related to cellular proliferation (31).

In the negative regulation of skeletal muscle regenerating, miR669a and miR669q can prevent skeletal muscle differentiation in postnatal cardiac progenitors (32). 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 (33). Sgcb-null cardiac progenitors spontaneously differentiated into skeletal muscle fibers both *in vitro* and when transplanted into regenerating muscles or infarcted hearts. Differentiation potential correlated with the absence of expression of a novel miRNA, miR669q, and with down-regulation of miR669a (32). In addition to negative regulator miR669a and miR669q, a negative feedback loop also reported. Lu and colleagues (34) identified a regulatory circuit between muscle miRNAs (including miR-1, miR-133 and miR-206) and Yin Yang 1 (YY1). YY1 is an epigenetic repressor of skeletal myogenesis in mouse and indeed represses muscle miRs expression in myoblasts and the repression is mediated through multiple enhancers and recruitment of Polycomb complex to several YY1 binding sites. They demonstrated that YY1 regulating miR-1 was functionally important for both C2C12 myogenic differentiation and injury-induced muscle regeneration. And further, miR-1 was in turn targets YY1, thus forming a negative feedback loop (34).

5. MICRORNAS IN KELETAL MUSCLE REGENERATION THROUGH REGULATING MYOD AND PAX GENES

Satellite cells located beneath the basal lamina of the muscle fiber, are normally maintained in a quiescent

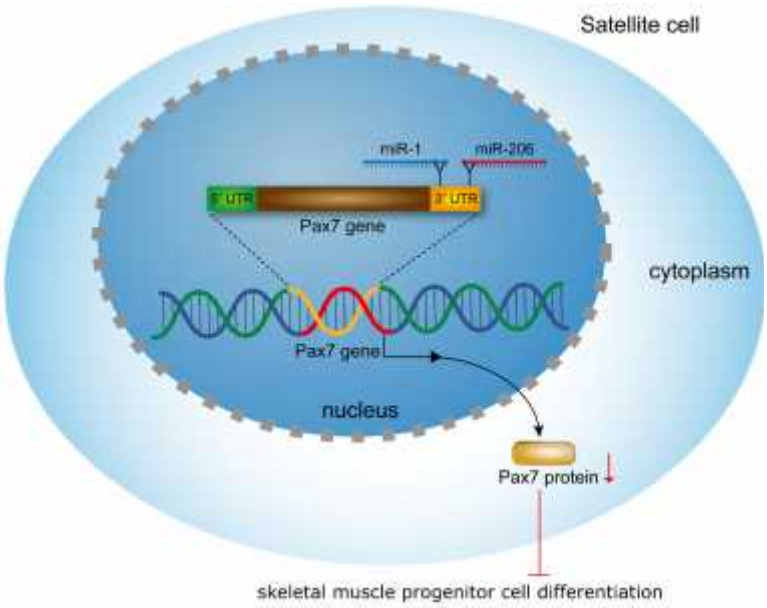

Figure 1. MicroRNA (miR)-1 and miR -206 regulate skeletal muscle progenitor cell differentiation by repressing Pax7. MiR-1 and miR-206 directly inhibit Pax7 gene expression by binding with sites in the 3' untranslated region (3' UTR) of Pax7, and then Pax7 protein level is decreased, resulting in restricting the progression of skeletal muscle progenitor cell differentiation.

state. when the muscle becomes damaged through injury or normal activity, they become activated. Satellite cells in the quiescent state express paired-box transcription factor 7 (Pax7) (35). Pax7 transcription factor is necessary for the biosynthesis, survival, and self-renewal of satellite cells. After activation the cells will express Pax7 and myogenic differentiation antigen (MyoD) 1 concurrently while the cells undergo a few rounds of division. These proliferating cells eventually withdraw from the cell cycle. Through downregulation of MyoD, they return to quiescence; or through downregulation of Pax7 and upregulation of myogenin, they differentiate. Thus expression of Pax7 and MyoD distinguishes the status of a cell, whether it is self-renewing (Pax7+/MyoD-), proliferating (Pax7+/MyoD+) or differentiating (Pax7-/MyoD+), respectively (36-38).

Scientists have already reported that MyoD activates the differentiation of skeletal muscle and stimulate microRNA-206 expression. Kim *et al.* (39) found that microRNA-206 could upregulate the MyoD gene by downregulating its inhibitory factor MyoR. This sort of positive feedback between microRNA and the regulatory factor for muscle generation and transcription could tilt the balance towards muscular differentiation, which could result in permanent differentiation of myocytes. In the diaphragm of the mdx mouse, the expression of MyoD1 was elevated 2.7-fold, meanwhile the expression of miR-206 was increased by 4.5-fold relative to control, consistent with a finding that MyoD1 can activate pri-miRNA-206 transcription (16). The study with local injection of dsmiR-1, miR-133 and 206 mixture showed that administration of exogenous miR-1, 133 and 206 can induce expression of MyoD1 and Pax7, as well as myogenin and myogenic markers, in mRNA and expression in the protein level at 3 and 7 days after injury, resulting in an morphological and physiological enhancement of muscle regeneration compared to the control siRNA (30). Thus, the combination of miR-1, 133 and 206 can promote not only myotube differentiation, but also the expression of MyoD1, myogenin and Pax7 were up-regulated in C2C12 cells *in vitro* (30). The study on the Duchenne muscular dystrophy (DMD) found that miR-206 can escape from the dystrophin-nNOS control being produced in activated satellite cells before dystrophin expression; in these cells, it contributes to muscle regeneration through repression of the satellite specific factor, Pax7 (22). Since Pax7 is regulated during the transition from proliferating satellite cells to differentiating myogenic progenitor cells, and miR-1 and miR-206 facilitate satellite cell differentiation by restricting their proliferative potential, the relationship between Pax7 and miR-1 and miR-206 has been studied. Chen and colleagues (40) identified Pax7 as one of the direct regulatory targets of miR-1 and miR-206. They demonstrated that inhibition of miR-1 and miR-206 substantially enhances satellite cell proliferation and increases Pax7 protein level with satellite cells isolated from mice. Conversely, sustained Pax7 expression as a result of the loss of miR-1 and miR-206 repression elements at its 3' untranslated region significantly inhibits myoblast differentiation (40). Thus, miR-1 and miR-206 directly inhibit Pax7 gene expression by binding with sites in the 3' untranslated region of Pax7, and then Pax7 protein level is decreased, resulting in restricting the progression of skeletal muscle progenitor cell differentiation (Figure 1).

As we mention above that during the differentiation of skeletal muscle, miR669a and miR669q was identified. And further, miR669a and miR669q were found to act upstream of myogenic regulatory factors to prevent myogenesis by directly targeting the MyoD 3'

untranslated region (32). MyoD also can up-regulate miR-378 during myogenic differentiation in C2C12 cells. ChIP and high throughput sequencing analysis shows that MyoD binds in close proximity to the miR-378 gene and causes both transactivation and chromatin remodeling. Overexpression of miR-378 increases the transcriptional activity of MyoD, in part by repressing an antagonist, MyoR. MyoR and miR-378 were anticorrelated during cardiotoxin-induced adult muscle regeneration in mice. Taken together, this shows a feed-forward loop where MyoD indirectly down-regulates MyoR via miR-378 (41).

MyoD(-/-) myoblasts display remarkable resistance to apoptosis by down-regulation of miR-1 and miR-206 and by up-regulation of Pax3, which resulte in transcriptional activation of antiapoptotic factors Bcl-2 and Bcl-xL. Hirai and colleagues found that forced MyoD expression induces up-regulation of miR-1 and miR-206 and down-regulation of Pax3, Bcl-2, and Bcl-xL along with increased apoptosis in MyoD(-/-) myoblasts. In contrast, MyoD gene knockdown increases cell survival of wild-type myoblasts. The 3' untranslated region of Pax3 mRNA contains two conserved miR-1/miR-206-binding sites, which are required for targeting of these miRNAs (42). Therefore, these data suggest that MyoD not only regulates terminal differentiation but also apoptosis through miRNA-mediated down-regulation of Pax3. During development, Pax3 is required for the maintenance of these cells in the somite and their migration to sites of myogenesis; high levels of Pax3 interfere with muscle cell differentiation, both in the embryo and in the adult. Crist and colleagues (43) identified that miR-27b directly targeted the 3' untranslated region of Pax3 mRNA as regulator. *In vivo* overexpression of a miR-27b transgene in Pax3-positive cells in the embryo leads to down-regulation of Pax3, resulting in interference with progenitor cell migration and in premature differentiation. Introduction of miR-27b antagomirs at a site of muscle injury *in vivo* also affects Pax3 expression and regeneration *in vivo*. Therefore, Pax3 protein level was regulated by miR-27b and this down-regulation ensures rapid and robust entry into the myogenic differentiation program (43).

Recently, besides of satellite cells, the importance of muscle side population (SP) cells located within the muscle interstitium has gained interest. Previous studies suggest that muscle SP cells may be precursors of the satellite cell population or may be independent progenitor cells that participate in muscle regeneration. MiRNAs expression in SP cells and main population (MP) cells in muscle were profiled by using quantitative real-time PCR-based expression assays. A set of miRNAs has been identified, which was highly expressed in SP cells as compared to MP cells. One miRNA, miR-128a, was found overexpressed in SP cells resulting in inhibited cell proliferation and differentiation potential, while it decreased in expression during continued culture *in vitro* (44). MiR-128a was found to regulate the target genes involved in the regulation of adipogenic-, osteogenic- and myogenic genes that include: Pax3, Runx1 and PPAR-gamma (44).

6. PATHWAYS AND MICRORNAS IN KELETAL MUSCLE REGENERATION

MiRNAs participates regeneration of skeletal muscle involving into some pathways. Mammalian target of rapamycin (mTOR) has emerged as a key regulator of skeletal muscle development by governing distinct stages of myogenesis. mTOR controls MyoD-dependent transcription of miR-1 through its upstream enhancer, most likely by regulating MyoD protein stability. Moreover, a functional pathway downstream of mTOR and miR-1 is delineated, in which miR-1 suppression of HDAC4 results in production of follistatin and subsequent myocyte fusion. Collective evidence strongly suggests that follistatin is the long-sought mTOR-regulated fusion factor. In summary, these findings unravel for the first time a link between mTOR and miRNA biogenesis and identify an mTOR-miR-1-HDAC4-follistatin pathway that regulates myocyte fusion during myoblast differentiation in vitro and skeletal muscle regeneration in vivo (45). By kinase-independent mammalian target of mTOR signaling, miR-125b biogenesis was negatively controlled both in vitro and in vivo as a part of a dual mechanism by which mTOR regulates the production of insulin-like growth factor 2 (IGF-II), a master switch governing the initiation of skeletal myogenesis (46).

In a study on DMD, platelet-derived growth factor receptor -beta, along with several other downstream targets of the phosphatase and tensin homolog deleted on chromosome 10/AKT (PTEN/AKT) pathway, as being modulated by miR-486. The generation of muscle-specific transgenic mice that overexpress miR-486 revealed that miR-486 alters the cell cycle kinetics of regenerated myofibers *in vivo*, as these mice had impaired muscle regeneration. These studies demonstrate a link for miR-486 as a regulator of the PTEN/AKT pathway in muscle regeneration (47).

7. CONCLUSIONS

MiRNAs play an important role in the regeneration of skeletal muscles. The emergence of the miRNA field provides an exciting opportunity to further understand the molecular factors which control skeletal muscle regeneration. miRNA biology also provides an avenue to dissect the mechanisms which may contribute to genetic and acquired muscle disorders and therapy on exercise-induced skeletal muscle injury. However, the mutual interaction mechanism regarding myogenic regulation factors and miRNAs remains unclear. *In vivo* studies on the functions and changes of miRNAs in the repair of skeletal muscular injury are rare, and studies on miRNAs during the repair of sports-induced skeletal muscle injury has not been reported. Nevertheless, as a new class of regulators of skeletal muscles regeneration, miRNAs hold the potential for the development of novel therapeutic strategies for muscular injury.

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Abbreviations: miRNAs:MicroRNAs; SP cells: side population cells; mdx: muscle of dystrophin; HDAC4, histone deacetylase 4; TIMP3: tissue inhibitor of metalloproteinase 3; TACE: TNF-alpha-converting enzyme; BDNF: brain-derived neurotrophic factor; ALS: amyotrophic lateral sclerosis; ds: double-stranded; siRNA, single-stranded RNA; YY1: Yin Yang 1; Pax: paired-box

transcription factor; DMD: Duchenne muscular dystrophy; MP cells: main population cells; IGF-II: insulin-like growth factor 2

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