REVIEW

Roles of miR-1, miR-133a, and miR-206 in calcium, oxidative stress, and NO signaling involved in muscle diseases

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MiR-1, miR-133a, and miR-206 are abundantly expressed in skeletal muscle and regulate the post-transcriptional expression of target genes. These miRNAs are upregulated in sera of DMD, BMD, LGMD, and FSHD patients, as well as *mdx* mice and CXMDj dogs, suggesting that the serum miRNAs may substitute for CK levels as be novel biomarkers for muscle disorders. These miRNAs are released into the extracellular environment in vesicular structures called exosomes, by mechanisms that are regulated by calcium, oxidative stress, and NO signaling. In this review, we will highlight the relationship between calcium, oxidative stress, and NO signaling and the release of miRNAs via exosomes as well as discuss the functions of these miRNAs.

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Introduction

miRNAs are evolutionally conserved non-coding single strand RNAs of approximately 19 - 23 nucleotides that regulate post-transcriptionally gene expression by binding to the 5'- or 3'-untranslated regions of target mRNAs [1,2] and thereby controlling their translation and/or mRNA degradation [3]. A single miRNA targets the expression of multiple genes, whereas one gene is regulated by multiple miRNAs [4]. The miRNAs miR-1 and miR-133a are expressed in cardiac and skeletal muscle, whereas miR-206/miR-133b is expressed only in skeletal muscle [5, 6]. The upregulation of miR-1, miR-133, and miR-206 levels during myoblast differentiation is known to protect myocytes against atrophy [7]. The expression of miR-1 is inhibited by the mTOR-specific inhibitor rapamycin and regulates myocyte fusion through the suppression of HDAC4 during myoblast differentiation *in vitro* and skeletal muscle regeneration *in vivo* [8]. MiR-1, miR-133, and miR-206 are upregulated in sera of DMD patients, *mdx* mice, and CXMDj dogs [9]. However, miR-1 and miR-133a expression is significantly downregulated by approximately two-fold in muscle tissue of *mdx* mice, whereas miR-206 is significantly upregulated [10]. Because the level of these miRNAs are less affected by exercise compared with creatine kinase levels, and furthermore they correlate with the level of motor activity of DMD patients [11], these miRNAs have been suggested as novel stable biomarkers for the diagnosis of and the outcomes of potential therapies for DMD. Moreover, as these miRNAs are upregulated in sera of BMD, LGMD, and FSHD patients [12], they have a potential as new biomarkers for muscle diseases.

MiRNAs release

In general, miRNAs are located in the intracellular- or extracellular space, depending on their consensus motif [13]. MiRNAs are released into the circulating blood encapsulated in exosomes, which are small membrane vesicles (approximately 100 nm in diameter) of late endosome origin

that are formed in the MVB, or are complexed with RNA-binding proteins, such as argonaute 2, high-density lipoprotein, and nucleophosmin 1, to protect their degradations by RNases [14-16]. Exosomes transport mRNAs, miRNAs, and proteins into target cells by their fusion and internalization by endocytosis into the target cells. The secretion of exosomes is induced by ceramide formation, which triggers the budding of intraluminal vesicles into the MVB [17, 18] and Rab GTPase family proteins regulate the budding, transport, motility, docking, and fusion of vesicles from the trans-Golgi network to the plasma membrane in a calcium-dependent manner [19-21].

Ca^{2+} signaling and miRNAs in muscle disorders

$Ca2^{+}$ influx from the extracellular compartment or increase in cytosolic Ca^{2+} in DMD patients are induced by membrane instability caused by the disruption of DGC in their muscles of DMD, leading to a worsening of their dystrophic phenotypes [22-26]. The main intracellular Ca^{2+} reservoir in mammalian cells is the ER, which contains three types of proteins that regulate intracellular Ca^{2+} concentration in the lumen, namely, 1) Ca^{2+} pumps that promote Ca^{2+} uptake, 2) Ca^{2+} -binding proteins that enable the store of Ca^{2+} in the ER lumen, and 3) Ca^{2+} channels that enable the release of Ca^{2+} into the cytosol [27]. IP3Rs are Ca^{2+} channels that are activated by the IP3-producing enzyme PLC, which leads to IP3-induced Ca^{2+} release [28-30]. In normal muscle, type I and II IP3Rs (IP3RI and IP3RII) are abundantly expressed in type II fibers, whereas the type III IP3R (IP3RIII) is distributed uniformly through muscles [31]. IP3RII protein level are significantly increased by approximately five fold in a DMD-derived muscle cell line, in which the slow Ca^{2+} signal activated through PLC that is induced by electrical stimulation was significantly faster than that in a control cell line [31]. IP3RII expression, which is indispensable in the heart but dispensable in skeletal muscle, is suppressed by miR-133a via SRF in both cardiac and skeletal muscle [32] and is suppressed in myocytes by alterations in the interaction or activity of SRF cofactors through IP3-induced calcium release [33].

Exosomal miRNAs

C2C12 myoblasts and myotubes were reported to secrete 0.37±0.15 and 0.41±0.23 [illegible] g/ml of exosomes into the medium, respectively [34]. Levels of miR-1, miR-133a, and miR-206 were reported to be upregulated in exosomes from C2C12 myotubes compared with those from myoblasts [34]. Exosomal miRNAs released from myotubes were found to repress Sirt1 gene expression in myoblasts [34]. In addition, exosomes from myotubes were found to reduce the proliferation of myoblasts and to induce their differentiation via the downregulation of Cyclin D1 and the upregulation of myogenin [34]. Larger amounts of muscle-enriched miRNAs in both exosomal and exosome-depleted supernatant fractions were detected in the serum of DMD patients compared with that in the controls [12]. Moreover, serum miR-133a levels in DMD patients were significantly increased in the exosomal fraction compared with the exosome-depleted supernatant fraction [12].

NO signaling and miRNAs in muscle diseases

The dysregulation of the levels of miRNAs cause abnormal muscle homeostasis, resulting in muscle diseases [35-37]. DMD [OMIM 310200] is an X-linked recessive progressive muscle disease with a prevalence of 1 in 3,500 live male births, and is caused by mutations in the dystrophin gene [3]. The dystrophin protein forms the DGC that integrates nNOS at sarcolemma, and produces NO, which inhibits the activity of HDAC2, a transcriptional repressor that acts via S-nitrosylation [38], which subsequently activates miR-1 and miR-133a [10]. The absence of dystrophin in DMD patients decreases the transcription of miR-1 and miR-133a by the binding of HDAC2 to their promoters in a NO-dependent manner [10]. On the other hand, miR-206 is upregulated in dystrophic muscle in a Dys/nNOS-independent pathway. The increase in miR-206 level may be important for the differentiation of satellite cells through the repression of Pax7 expression, because miR-206 is present in immature regenerating and differentiated muscle in *mdx* mice [39]. Such abnormal expression of these mRNAs in *mdx* mice return to normal levels similar to those of wild-type mice by dystrophin rescue [10]. In *mdx* mice and DMD patient muscle, the miR-1 and miR-206 levels are significantly downegulated and upregulated, respectively [40]. Such dysregulated miRNA levels are restored to those of wild-type mice by class I HDAC inhibition and eNOS expression [40].

MiRNAs in myoblast proliferation and differentiation

The expression levels of miR-1 and miR-133a are upregulated in differentiated C2C12 myoblasts and myotubes [5]. Both miR-1 and miR-133a play roles in the *in vitro* differentiation and proliferation of myoblasts through the repressions of HDAC4 and SRF, respectively, in differentiation medium [5]. On the other hand, miR-133a represses myoblast proliferation and promotes the differentiation of myoblasts into myotubes by inhibition of the ERK1/2 pathway through direct silencing of FGFR1 and PP2AC genes, in growth medium [41]. Furthermore, miR-133a expression is repressed by ERK1/2 activation, and moreover, miR-133a is involved in a feedback circuit regulating the ERK1/2 pathway, which controls myoblast

proliferation and differentiation [41]. In addition, the expression of miR-133a inhibits the proliferation of C2C12 myoblasts and promotes their differentiation at an early stage in the differentiation process, through direct suppression of translation of the UCP2 gene, a regulator of energy expenditure and thermogenesis [42], or the production of muscle-specific transcripts by targeting alternative splicing factor, nPTB [43]. The levels of miR-1, miR-133, and miR-206 are upregulated during muscle regeneration in mice after muscle injury [39,12] Mice lacking miR-133a show centronuclear myopathy in type II fast-twitch myofibers, accompanied by impaired fast-to-slow myofiber conversion, increased oxidative enzyme activity, and increased expression of the miR-133a target gene, dynamin2, which is a large GTPase implicated in the regulation of actin and microtubule cytoskeletons [44]. Recently, it was reported that miR-133a promotes slow-to-fast myofiber conversion through the indirect downregulation of MyHC-I gene expression, via the inhibition of the TEAD1 transcriptional factor by thyroid hormone signaling [45].

Although a low-dose of the synthetic glucocorticoid DEX protects muscle cells from atrophy through the suppressions of pro-inflammatory cytokines, high doses of DEX induce muscle atrophy by the inhibition of IGF-I signaling through a decrease of PI3K activity [46-50]. The expression of IGF-IR is directly suppressed by miR-133a, which leads to the inhibition of PI3K/Akt signaling by a negative feedback loop [51]. The long non-coding pre-RNA, linc-MD1, which enhances miR-133 function by acting as a ceRNA via binding to it and hence inhibiting HuR expression, controls the early phases of myogenesis [52]. In addition, the overexpression of the miR-133a in the presence of the reprogramming factors, Gata4, Mef2c, and Tbx5, leads to the efficient generation of beating iCMs, via the direct repression Snai1, a master regulator of epithelial-to-mesenchymal transition [53].

Muscle disorders associated dysregulated exosome secretion

Exosomes carrying miRNAs are secreted by activation of the Ca^{2+} signal pathway, which regulates the docking and fusion of MVBs into the plasma membrane through SNAREs [19, 20, 54-56], or S1P/ceramide pathway [17, 57-59], in which the ceramide synthesis enzyme, *Smpd3*, is activated by oxidative stress [60-63], resulting in the secretion of exosomes [64]. The absence of dystrophin in *mdx* mice induces an abnormal increase of calcium influx in the sarcolemma of adult skeletal muscle fibers, resulting in muscle degeneration [65-69]. In addition, the loss of dystrophin inhibits NO signaling by preventing the associations of the DGC with nNOS, which contributes to the muscle atrophy [70-74]. The eNOS pathway negatively regulates the secretion of exosomes [75]; thus, an increase of exosome secretion into the serum from muscle tissue in DMD patients might be caused by calcium, oxidative stress, or NO signaling. FSHD (OMIM158900) is an autosomal-dominant neuromuscular disorder with a prevalence of approximately one per 20,000 in the Japanese population [76], is caused by a loss of a stretch of microsatellite repeats approximately 3.3 kb in length in the D4Z4 on chromosome 4q35 [77], which contains a functional promoter for DUX4 and DUX4c [78, 79] This muscle disorder is characterized by the overexpressions of DUX4 and DUX4c in the muscle, which induces the expressions of miR-1, miR-133a, and miR-206 [80]. Myoblasts from FSHD patients show increased susceptibility to oxidative stress [81-83], which enhances the release of exosomes [64]. LGMD is caused by one of a total of 22 autosomal dominant or recessive gene mutations, and has an incidence of about one per 20,000 individuals [84]. Of the various types, LGMD2A is caused by a mutation in the enzyme calpain 3, resulting in increased oxidative stress in the muscles of patients with this disease [85]. Therefore, oxidative stress in the muscles of FSHD and LGMD2A patients might regulate the secretion of exosomes into the extracellular environment.

Conclusions

Increasing lines of evidence indicate that miRNAs that are abundant in the muscle play pivotal roles in the regulations of myogenesis, and are associated with muscle disorders. The secretion of miRNA-containing exosomes is regulated by the calcium, oxidative stress, and NO signaling pathways, which are all dysregulated in muscle disorders. Exosomes secreted into the extracellular space can communicate with target cells through the transfer of miRNAs. Therefore, not only the miRNAs, but also the exosomes are potential targets for new diagnostic tools and novel therapies for muscle disorders.

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