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Chapter

miRNAs and Muscle Stem Cells

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Abstract

Skeletal muscle represents between 30 and 38% of the human body mass. Both the maintenance and repair of adult muscle tissue are directed by satellite cells (SCs). SCs are located beneath the basal lamina of the skeletal muscle myofiber. They are quiescent for most of their life but, in response to physiological stimuli or muscle trauma, they activate, proliferate, and enter the myogenic program via generating myogenic progenitors (myoblasts) that fuse to existing myofibers or *de novo* myofibers. MicroRNAs (miRNAs or miRs) play a critical role in regulating muscle regeneration and stem cell behavior. In this chapter, we review the pivotal role in the regulation of SC quiescence, activation, and differentiation in the context of muscular dystrophies.

Keywords: microRNA, satellite cell, quiescence, myogenesis, muscle regeneration, muscular dystrophies

1. Introduction

With more than 600 individual muscles in humans, skeletal muscle tissue represents between 30 and 38% of the human body mass [1]. This tissue is essential not only to provide ambulatory capacity to our organism but also to control such important functions as breathing and thermogenesis. Although its composition is heterogenous, each single skeletal muscle is mainly composed by individual muscle fibers consisting of elongated multinucleated syncytia. These myofibers are diversified in size, shape, and contractile protein content to fulfill the different functional needs of our body. This tissue retains a highly adaptive and robust capacity to regenerate throughout most of life, thanks to the presence of a stem cell-like population termed as satellite cells (SCs) [2].

miRNAs have emerged as critical regulators of numerous biological processes by modulating gene expression at the posttranscriptional 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. In that context, the role of *miRNAs* in SC biology is beginning to be explored. In this chapter, we will focus in our understanding of how miRNAs act in controlling the ability of SCs to appropriately balance SC function during muscle regeneration as well as in the context of neuromuscular diseases.

2. Basic biology of SCs

SCs, originally identified via electron microscopy in 1961 by Alexander Mauro, are located underneath the basal lamina and adjacent to the plasma membrane of the skeletal muscle myofiber [3]. It has been established that SCs in adult muscle represent a lineage continuum of embryonic myogenic progenitor cells. SCs of the body and limbs arise from somites, in common with the muscle that they are associated with [4–6], while SCs located in head muscles also originate from the cranial mesoderm [7]. In undamaged muscle, the majority of *satellite cells* are *quiescent*, characterized by the expression of the transcription factor PAX7 [8]. Within a context of physiological stimuli (physical exercise or pathological conditions), SCs become activated and enter into the cell cycle to expand their progeny and form myogenic precursor cells or myoblasts [8]. SCs' activation is mediated by the induced expression of myogenic factor 5 (MYF5) and myogenic determination protein (MYOD) [2]. The differentiation of myogenic committed cells involves downregulation of PAX7 and *de novo* expression of myogenin (MYOG), which is followed by fusion of the newly formed differentiated myoblasts among them and with the remaining myofibers to repair damaged muscle [2]. In addition to providing myogenic precursors, activated SCs also undergo self-renewing proliferation that replenishes the pool of muscle SCs, thereby ensuring that the capacity to respond to future injuries is maintained in the muscle [2].

3. Biogenesis of miRNAs

Canonical miRNAs are transcribed by RNA polymerase II into primary transcripts called pri-miRNA, bearing 5′m7G cap and 3′ poly(A) tail structures [9]. Sometimes, miRNA loci can comprise no single but multiple and overlapping miRNA genes, called clusters, which are processed from the same polycistronic primary transcript [10]. Once transcribed, the pri-miRNA forms a stem-loop structure. Then, the RNA-binding protein Di George syndrome critical region gene 8 (DGCR8) recognizes it and directs the nuclear RNase III enzyme endonuclease, DROSHA, toward the pri-miRNA. DROSHA cleaves at the base of the hairpin embedded within the pri-miRNA [11], yielding a ~70-nt hairpin molecule termed precursor miRNA or pre-miRNA. Soon after, the pre-miRNA is transported from the nucleus by exportin 5 to the cytoplasm via a Ran-GTP-dependent mechanism [12]. Once in the cytoplasm, a second RNase III endonuclease, DICER, cleaves the pre-miRNA, thus removing the terminal loop of the pre-miRNA and releasing a mature double-stranded ~22-nt miRNA molecule [13]. One strand of this duplex RNA molecule (the guide strand) is transferred to the RNA-induced silencing complex (RISC) containing argonaute 2 (AGO2) and the RNA-binding protein TARBP2 [TAR (HIV) RNA-binding protein 2], while the other is degraded [14]. The miRNA's function at this time is to guide the silencing complex to the target mRNA through complementary binding of the miRNA seed sequence, which results in inhibition of translation and/or degradation of the target transcript [15] (**Figure 1**).

Approximately, half of vertebrate miRNAs are processed from introns of protein-coding genes or genes encoding for other ncRNA classes, for instance, small nucleolar RNA (snoRNAs) or long intervening noncoding RNAs (lincRNAs) [10]. The biosynthesis of these miRNAs bypass one or more steps in the canonical biogenesis pathway, being therefore termed noncanonical miRNAs. In this sense, it is important to stress that while DROSHA and DGCR8 are only needed to process canonical miRNAs, DICER is almost always indispensable in the production of both canonical and noncanonical miRNAs [16]. Among noncanonical miRNAs, the most

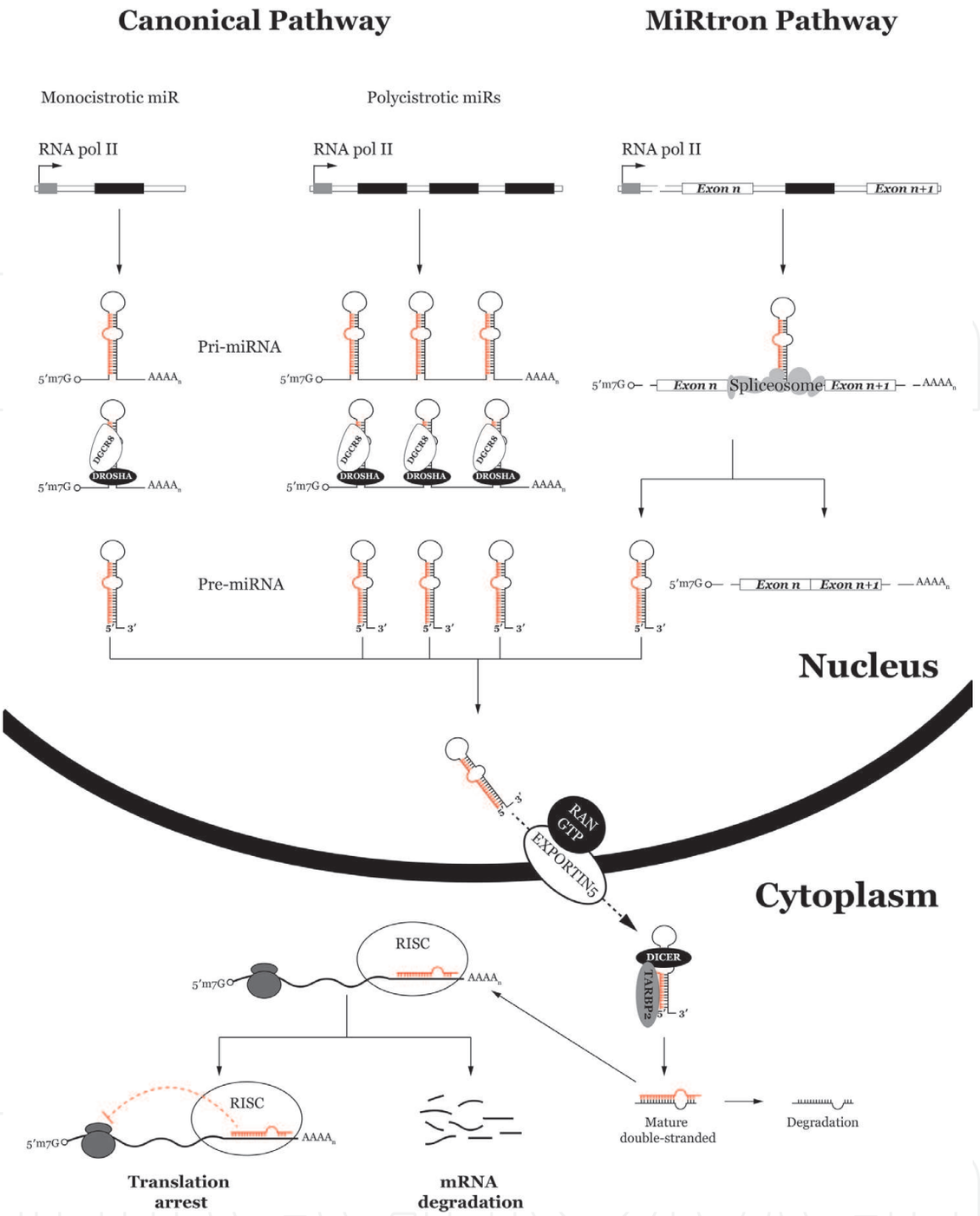

Figure 1.
Schematic representation of biogenesis of miRNAs.

studied have been those so-called miRtrons, located within intron sequences. The expression profiles of these miRNAs coincide with the transcription of their host genes [10], being released from the excised host introns by the spliceosome [17, 18] in a typical mirtron-maturing fashion (**Figure 1**).

4. miRNAs and SC function

Deep sequencing analyses have shown that many miRs are expressed in muscle tissue [19–21]. Among these miRs, there is a group, so-called myomiRs, whose expression is restricted to muscle tissue. This family is composed by miR-1, miR-133a, and miR-206, miR208a, miR-208b, miR-486, and miR-499 [22–28]. While

most myomiR family members are expressed in both the heart and skeletal muscle, miR-208a is cardiac-specific and miR-206 is skeletal muscle-specific. A deeper analysis of these family members is well reviewed in [29]. Nevertheless, not only myomiRs but also other miRs with a more ubiquitous expression play important roles in the muscle. It has also been shown that miRNAs are essential for muscle homeostasis and regeneration upon injury, since either systemic or conditional deletion of DICER in muscle PAX7^{+} population results in a depletion of SCs and a quasi-absence of repair upon injury cell [30]. In addition, to date, *in vitro* and *in vivo* experiments have shown that many miRs expressed in the skeletal muscle rule quiescence, activation, proliferation, fate specification, and differentiation of muscle progenitor cells by regulating the expression of myogenic differentiation regulators, transcription factors, structural proteins, and cytoskeletal components that are required to give rise to the differentiated muscle phenotype. In this section, we will review some of them, analyzing their roles in quiescence, activation-proliferation, and differentiation estates.

4.1 Control of the quiescence state

In vivo, SCs are normally in a quiescent state after the postnatal development. Cheung et al. showed that the quiescent state is strongly controlled by miRs, since SCs lacking a functional *Dicer* gene, spontaneously exit from the quiescent state [30]. In this work, the authors demonstrate that miR-489 regulates SCs quiescence in a cell-autonomous manner through the control of the oncogene *Dek* (DEK proto-oncogene), whose protein is not expressed in quiescent SCs (QSCs) but is strongly upregulated after SC activation. Soon after, Crist et al. showed that miR-31 targets *Myf5* mRNA in QSCs, thus preventing MYF5 protein accumulation and premature activation of these muscle stem cells [31]. miR-31 is sequestered with *Myf5* transcripts in cytoplasmic mRNP granules in QSCs and, upon SCs' activation, these mRNPs rapidly dissociate and relieve the spatial constraint on miR-31 and Myf5 mRNA, allowing the rapid translation of the MYF5 protein. Recently, Baghdadi et al. have added a new miR to the list of miRs that control the quiescent state of SCs [32]. In this work, the authors shown that miR-708 regulates quiescence and self-renewal by active repression of SC migration. Notch signaling is directly implicated in this control by inducing transcription of miR-708 that represses Tensin3 (*Tns3*), a component of the focal adhesion complex. This repression inhibits focal adhesion kinase (FAK) activation, which in turn stabilizes SCs within their niche (**Table 1** and **Figure 2**).

4.2 Activated proliferative state

Early evidences of miRs controlling proliferation in myoblast were reported by Chen et al. in 2006 [33]. In this work, the authors showed that miR-133 enhances myoblast proliferation by repressing the serum response factor (Srf) *in vitro* and *in vivo* in *Xenopus laevis* embryos. Similarly, Cai et al. have recently shown that miR-664 also promotes myoblast proliferation by targeting *Srf* mRNA [34]. Other miR that proposed to promote myoblast proliferation is miR-27. Huang et al. showed that miR-27, for this purpose, targets myostatin (Mstn), a well-known negative regulator of myogenesis [35]. Sometimes, miR members of a same cluster can work together in order to achieve the same biological effect. In this sense, Qiu et al. have shown that miR-17, miR-20a, and miR-92a, three members of the miR-17-92 cluster, repress PDZ and LIM domain 5 (Pdlim5), also known as Enh1 expression at heart and skeletal muscle. This protein exerts antiproliferative effects in myoblast. Thus, its inhibition contributes to promote myoblast proliferation and prevents differentiation [36] (**Table 1** and **Figure 2**).

microRNAs	Targets	Function	References
miR-489	*Dek*	Regulates SCs' quiescence	[30]
miR-31	*Myf5*	Prevents MYF5 protein accumulation and premature activation of SCs	[31]
miR-708	*Tns3*	Regulates quiescence and self-renewal by active repression of SCs' migration	[32]
miR-133	*Srf*	Enhances and/or promotes myoblast proliferation	[33]
miR-664	*Srf*	Enhances and/or promotes myoblast proliferation	[34]
miR-27	*Mstn*	Enhances and/or promotes myoblast proliferation	[35]
miR-17, miR-20a, and miR-92a	*Pdlim5*	Enhance and/or promotes myoblast proliferation	[36]
miR-195 and miR-497	*Igf1r, Insr, Ccne1, and Ccnd2*	Inhibit myoblast proliferation	[37]
miR-487b	*Irs1*	Inhibits myoblast proliferation	[38]
miR-16	*Foxo1*	Suppresses myogenesis	[39]
miR-1 and miR-133	*Ccnd1 and Sp1*	Inhibit myoblast proliferation	[40]
miR-15b, miR-23b, miR-106b, and miR-503	*Ccnd1 and Ccnd2*	Keep SCs in a quiescent state	[41]
miR-106b	*Myf5*	Keeps SCs in a quiescent state	[41]
miR-1	*Hdac4*	Promotes myoblast differentiation	[33]
miR-1 and miR-206	*Pax7*	Restrict myogenic progenitor cell proliferation and promote differentiation	[42]
miR-206	*Pax7*	Activates myoblast differentiation	[43]
miR-206	*Pax7, Notch3, and Igfbp5*	Stimulates SC differentiation and skeletal muscle regeneration	[44]
miR-206	*Hdac4 and Pola1*	Promotes myoblast differentiation and induces a cell cycle arrest	[45]
miR-1 and miR-206	*Gja1*	Promote myoblast fusion	[46]
miR-206 and miR-486	*Pax7*	Promote initial muscle differentiation	[47]
miR-486	*Pten, Pdgfrβ, Foxo1, Sfrs1, and Sfrs3*	Promotes myoblast differentiation	[48–50]
miR-133	*Fgfr1 and Pp2ac*	Promotes muscle precursor cells differentiation	[51]
miR-29	*Rybp and Yy1*	Ensures proper myoblast differentiation into myotubes	[52]
miR-29	*Hdac4*	Promotes myoblast differentiation	[53]
miR-29	*Akt3*	Reduces proliferation and facilitates differentiation of precursor muscle cells	[54]
miR-26a	*Ezh2*	Induces muscle cell differentiation	[55]
miR-26a	*Smad1 and Smad4*	Promotes myoblast differentiation	[56]
miR-214	*Ezh2*	Promotes myoblast differentiation	[57]
miR-214	*N-ras*	Promotes myogenic differentiation by facilitating exit from mitosis	[58]

microRNAs	Targets	Function	References
miR-181	*HoxA11*	Promotes myogenic differentiation	[59]
miR-378	*MyoR*	Promotes myogenic differentiation	[60]
miR-205a	*Cdh11*	Inhibits myoblast proliferation and promotes myoblast differentiation	[61]
MiR-675-3p	*Smad1 and Smad5*	Promotes myogenic differentiation by repression of BMP pathway	[62]
miR-675-5p	*Cdc6*	Promotes myogenic differentiation by repression of DNA replication	[62]
miR-17	*Ccnd2, Jak1, and Rhoc*	Promotes differentiation of precursor muscle cells	[63]
miR-34b	*Igfbp2*	Represses proliferation and promotes differentiation of myoblasts	[64]
miR-664	*Wnt1*	Downregulates WNT signaling to allow for normal myogenic differentiation to occur	[34]
miR-199a	*Wnt2, Fzd4, and Jag1*	Downregulates WNT signaling to allow for normal myogenic differentiation to occur	[65]
miR-155	*Mef2a*	Represses myoblast differentiation	[66]
miR-351	*Lactb*	Represses myoblast differentiation	[67]
miR-23a	*Myh1, Myh2, and Myh4*	Prevents myogenic differentiation	[68]

Table 1.
General overview of miRNAs involved in adult myogenesis.

miRNAs can modulate negative proliferation in myoblast as well. In this sense, Wei et al. showed that the protein complex NF-κB can induce miR-195 and miR-497 expression, thus inhibiting myoblast proliferation by targeting insulin-like growth factor I receptor (Igf1r), insulin receptor (Insr), cyclin E1 (Ccne1), and cyclin D2 (Ccnd2) mRNAs [37]. Thus, NF-κB inhibition must be accomplished to induce proliferation in these cells. Another miR that targets insulin signaling proteins is miR-487b. This miR represses insulin receptor substrate 1 (Irs1) mRNA, thus exerting a negative control of myoblast proliferation [38]. Similarly, it has been reported that miR-16 acts as a coordinated mediator that can suppress myogenesis in avian hypertrophic skeletal muscles through the control of myoblast proliferation by targeting forkhead box O1 (Foxo1) mRNA [39], a transcription factor that governs muscle growth, metabolism, and cell differentiation [69]. Growth factors such as fibroblast growth factors (FGFs) regulate cell proliferation and differentiation in numerous tissues, including skeletal muscle [70]. In this sense, Zhang et al. [40] have shown that FGF2 released from the myotrauma represses p38 signaling and expression of miR-1 and miR-133. Thus, the repressed p38 signaling and subsequent downregulation of miR-1 and 133 induce an upregulation of their respective targets, cyclin D1 (Ccnd1) and Sp1 transcription factor (Sp1), that jointly facilitate the SC proliferation at the early stages of muscle regeneration. Nevertheless, this work contradicts the pro-proliferating role for miR-133 proposed by Chen et al. in 2006 [33]. Our group has also shed light over this issue. Thus, Lozano-Velasco et al. unravel the existence of a Pitx2-miRNA pathway that modulates cell proliferation in myoblasts and skeletal muscle SCs [41]. In this work, we demonstrated that miR-15b, miR-23b, miR-106b, and miR-503 keep SCs in a quiescent state by targeting Ccnd1 and Ccnd2. Once QSCs are activated, Pitx2c is upregulated and exerts a

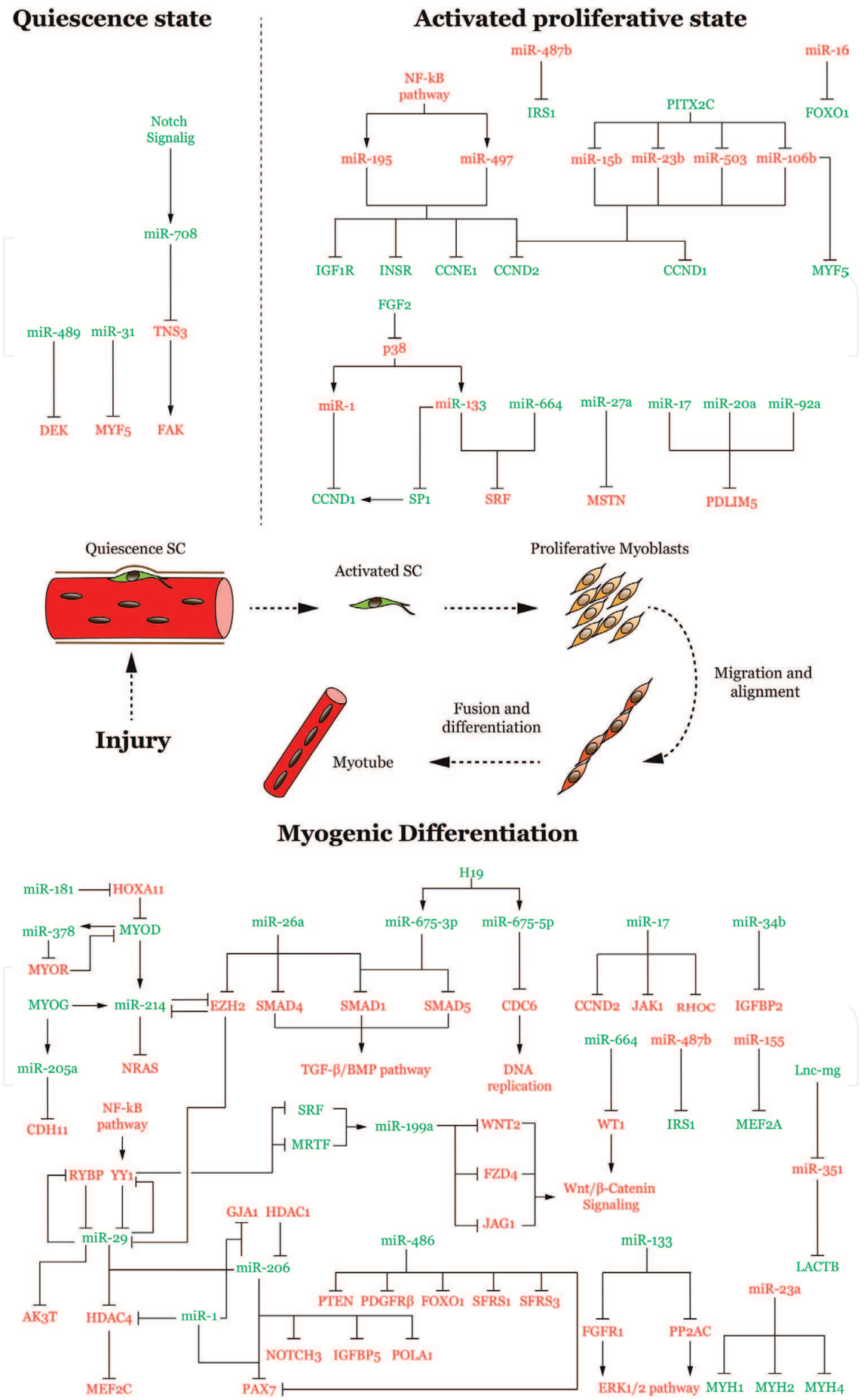

Figure 2.
miR controlling differentiation of muscular precursor cells. Green and red labels correspond with the induced or repressed molecules on each state, respectively (quiescent state, activated proliferative state, or myogenic differentiation).

repressive effect over miR-15b, miR-23b, miR-106b, and miR-503 promoters, thus allowing Ccnd1 and Ccnd2 mRNA to be translated. On the other hand, this Pitx2 upregulation also avoids the repressive effect of miR106b over Myf5 mRNA, thus promoting myoblast commitment to a myogenic cell fate (**Table 1** and **Figure 2**).

4.3 Myogenic differentiation

SC differentiation is a complex process. In this stage, the cells need to switch off proliferative signals and upregulate structural genes turning simple individual cells into a complex syncytium with the ability to coordinately contracts. In this scenario, miRs have also been described as essential molecules. Focusing on myomiRs, Chen et al. showed that miR-1 promotes myoblast differentiation by targeting histone deacetylase 4 (Hdac4) mRNA, a transcriptional repressor of muscle gene expression [33]. HDAC4 has been shown to inhibit muscle differentiation and skeletal muscle gene expression, mainly by repressing myocyte enhancer factor 2C (MEF2C), an essential muscle-related transcription [71]. The same group also showed that miR-1 and miR-206 restrict myogenic progenitor cell proliferation and promote differentiation by directly downregulating Pax7 expression [42]. At the same time, Cacchiarelli et al. also showed that miR-206 activates myoblast differentiation through Pax7 repression at early stages of differentiation [43]. These authors have shown that, in SCs, miR-206 is specifically repressed by histone deacetylase 1 (HDAC1), but under differentiation conditions, repressive effect over miR-206 promoter mediated by HDAC1 disappear thus allowing Pax7 repression and promoting myoblast differentiation [43]. Soon after, Liu et al. showed that loss of miR-206 results in upregulation of Pax7, notch receptor 3 (Notch3), and insulin-like growth factor-binding protein 5 (Igfbp5) in differentiating miR-206 KO SCs compared with WT cells, implying that repression of these inhibitors of myogenesis accounts, at least in part, for the stimulatory influence of miR-206 on SC differentiation and skeletal muscle regeneration [44]. As miR-1, miR-206 also promotes myoblast differentiation by targeting Hdac4 [53] and induces a cell cycle arrest through the repression of DNA polymerase alpha 1 (Pola1, catalytic subunit), a specific subunit of DNA polymerase α [45]. miR-1 and miR-206 also work coordinately, downregulating gap junction protein (Gja1, alpha also known as Connexin 43) expression during myoblast fusion as Anderson et al. had shown previously [46]. Dey et al. also corroborated that miR-206 targets Pax7 mRNA [47] and, in the same work, they also demonstrated that miR-486 exerts the same effect in order to promote initial muscle differentiation. Regarding this miR, Alexander et al. showed that miR-486 targets phosphatase and tensin homolog (Pten), platelet-derived growth factor receptor beta (Pdgfrβ), Foxo1, serine and arginine-rich splicing factor 1 (Sfrs1), and serine and arginine-rich splicing factor 3 (Sfrs3) mRNAs. Proteins derived from these mRNAs comprise the PTEN/AKT pathway, which is essential for normal cellular proliferation [48–50]. Thus, miR-486 overexpression and consequent PTEN/AKT pathway inhibition are required for proper myoblast differentiation as well. A role for miR-133 in myogenic differentiation has also been proposed. Thus, Feng et al. showed that miR-133 promotes muscle precursor cells differentiation by downregulating two members of the pro-proliferation ERK1/2 signaling pathway, fibroblast growth factor receptor 1 (Fgfr1), and protein phosphatase 2 (Pp2ac, catalytic subunit, alpha isozyme) [51]. All together, these data bring out the important role carried out by myomiRs during early steps of muscle differentiation (**Table 1** and **Figure 2**).

Focusing on non-muscle-specific miRs involved in myogenic differentiation, another miR with a relevant importance during muscle differentiation is miR-29. miR-29 seems to promote myogenesis by downregulating multiple targets related

to the NF-kB signaling pathway. In this sense, Wang et al. unraveled a myogenic circuit that involves constitutive activity of NF-kB in myoblasts regulating the YY1 transcription factor, which subsequently suppresses the miR-29 promoter activity by recruiting the enhancer of zeste homolog 2 (histone methyltransferase, EZH2) as well as the histone deacetylase protein HDAC1, thus maintaining cells in an undifferentiated state [72]. In this regard, Zhou et al. later showed that miR-29 is able to directly target the RING1- and YY1-binding protein (Rybp) [52]. RYBP is a negative regulator of skeletal myogenesis, which, together with EZH2 and HDAC1, functions as a corepressor of YY1 to silence miR-29 promoter [52]. Thus, as differentiation ensues, downregulation of the NF-kB-YY1 pathway, RYBP, and EZH2 lead to upregulation of miR-29 that in turns further decreases YY1 and Rybp levels to ensure proper differentiation into myotubes. As miR-1 and miR-206, miR-29 also promotes myoblast differentiation by targeting Hdac4 [53]. In addition, miR-29 targets AKT serine/threonine kinase 3 (Akt3), a member of the serine/threonine protein kinase family responsive to growth factor cell signaling, to reduce proliferation and facilitate differentiation of precursor muscle cells in skeletal muscle development [54] (**Table 1** and **Figure 2**).

Another non-muscle-specific miR whose expression is upregulated during myogenic differentiation is miR-26a. The important role of miR-26 in inducing muscle cell differentiation had also been previously demonstrated by Wong et al., who showed that miR-26 acts to posttranscriptionally repress Ezh2, a known suppressor of skeletal muscle cell differentiation that belongs to the polycomb group (PcG) of proteins that suppress gene transcription through histone methylation, thus promoting miR-29 promoter activity as we have previously indicated [55]. In addition, Dey et al. showed that miR-26a directly targets SMAD family member 1 (Smad1) and SMAD family member 4 (Smad4), two critical transcription factors that belong to TGF-β/BMP pathway, whose activity inhibits myogenesis [56] (**Table 1** and **Figure 2**).

miR-214 upregulation is also required for myogenesis. During SCs' activation and proliferation, EZH2 is highly expressed in the generated myoblasts, thus allowing PcG proteins to repress transcription from the intronic region containing miR-214 [57]. The initial phase of cell differentiation is characterized by reduced Ezh2 expression and consequent derepression of the miR-214 locus. Then, in a negative feedback, miR-214 targets Ezh2 mRNA, thus reducing its translation [57]. At this point, the continuous PcG disengagement leads to recruit MyoD/MyoG to the miR-214 promoter, thus enhancing its transcription [57]. This negative feedback, together with miR-26a via Ezh2, contributes to enhance miR-29 promoter activity. On the other hand, miR-214 is also able to promote myogenic differentiation by facilitating exit from mitosis via downregulation of neuroblastoma ras oncogene (N-ras) [58]. Regarding to the effect that MyoD exerts over miR-214 promoter, Naguibneva et al. demonstrated that homeobox A11 (HoxA11), a negative regulator of MyoD expression, is a direct target of miR-181 during mammalian muscle differentiation. Thus, under differentiation conditions, miR-181 is upregulated, resulting in downregulation of HoxA11 and the consequent release of MyoD expression [59]. MyoD also binds in close proximity to the miR-378 gene and causes its transactivation [60]. Parallelly, this miR targets MyoR mRNA, thus avoiding the antagonist effect of MYOR over MYOD, constituting a feed-forward loop where MyoD indirectly downregulates MyoR via miR-378 [60]. Besides, by using chicken myoblasts, Wang et al. have described how miR-205a is regulated by myogenin (MyoG) transcription factor, which can bind to the promoter region of miR-205a gene in chicken, thus inducing its expression. The upregulation of miR-205a can inhibit myoblast proliferation and promote myoblast differentiation by its repression on cadherin-11 (CDH11), a crucial regulator of postnatal skeletal growth [61] (**Table 1** and **Figure 2**).

H19 long noncoding RNA and its encoded miRNAs, miR-675-3p, and miR-675-5p are expressed in the skeletal muscles and also are upregulated during myoblast differentiation and muscle regeneration [62]. Dey et al. have shown that MiR-675-3p targets Smad1 and SMAD family member 5 (Smad5) mRNAs, while miR-675-5p represses cell division cycle 6 (Cdc6) mRNA. Consequently, through SMAD1 and SMAD5 proteins' downregulation, miR-675-5p induces a repression of BMP pathway as well as a repression of DNA replication through CDC6 protein downregulation, thus promoting myoblast differentiation [62]. Similarly, Kong et al. have also shown that miR-17 targets Ccnd2, Janus kinase 1 (Jak1) and ras homolog family member C (Rhoc) mRNAs. These genes are critical for cell proliferation and/or fusion, hence their inhibition promotes differentiation of precursor muscle cells [63]. In this sense, Wang et al. have also shown that miR-34b represses the proliferation and promotes the differentiation of myoblasts by targeting insulin-like growth factor-binding protein 2 (IGFBP2) [64] (**Table 1** and **Figure 2**).

miR-664 induces myogenic differentiation through targeting Wnt family member 1 (Wnt1), hence blocking the canonical Wnt/β-catenin signaling pathway [34]. In this regard, Alexander et al. unraveled a SRF/MRTF-dependent mechanism for the induction of miR-199a transcription during myoblast differentiation [65]. In this stage, miR-199a represses WNT2, FZD4, and JAG1 and subsequently downregulates WNT signaling to allow for normal myogenic differentiation to occur [65]. In this work, the authors also indicate that, in previous stages, miR-199a-5p transcription is likewise repressed by YY1, as happened with miR29 promoter (**Table 1** and **Figure 2**).

miRNAs can also negatively modulate myoblast differentiation. Thus, it has been shown that miR-487b must be downregulated in order to avoid its suppressive effect over Irs1 mRNA, as happens during proliferation stage [38]. The MEF2 proteins are transcription factors that act in conjunction with myogenic regulatory factors (MRFs) to regulate muscle differentiation [73]. In this sense, Seok et al. showed that miR-155 represses myoblast differentiation by repressing Mef2a mRNA, hence miR-155 downregulation is necessary to prevent Mef2a downregulation and to induce a proper myoblast differentiation [66]. In a newfangled fashion lnc-mg, a long noncoding RNA that promotes myoblast differentiation [74] has been described to act as a competing endogenous RNA (ceRNA) sponging miR-351, thus reducing the effect of miR-351 on its direct target lactamase-β (LACTB) to promote myoblast differentiation [67] (**Table 1** and **Figure 2**).

MiRs are also capable to regulate structural proteins needed at last stages of myoblast differentiation. Regarding this, Wang et al. showed that miR-23a prevents myogenic differentiation through downregulation of fast myosin heavy chain isoforms. Thus, downregulation of miR-23a during final steps of muscle differentiation allows myotubes to express the myosin heavy chain genes Myh1, Myh2, and Myh4 [68] (**Table 1** and **Figure 2**).

5. miRNAs and muscle cells in muscular dystrophies

Primary muscular disorders are the consequence of a disease that directly affects skeletal muscle [75]. Among them, the most important group, in terms of number of people affected as well as economic impact generated in the developed world, are muscular dystrophies. These pathologies are inherited myogenic disorders characterized by progressive muscle wasting and weakness of variable distribution and severity [76]. The genes and their protein products that cause most of these disorders have now been identified [76]. However, miRNAs misregulation related to them still remains poorly understood. In this section, we focus in the understanding

of how miRNAs act in regulating muscle cells in the context of Duchenne muscular dystrophy (DMD) and myotonic dystrophy (DM), the most common inherited muscle diseases of childhood and adulthood, respectively. In addition, we discuss current miR-related molecular diagnosis and therapy approaches implemented in the field in order to ameliorate the progression of these pathologies by modulating muscle precursor biology.

5.1 miRNA in muscle precursor cells in the context of muscular dystrophies

DMD is the most severe form of muscular dystrophies. It is the most common inherited muscle disease of childhood afflicting approximately 1 in 3500 young males [77]. It is characterized as a muscular disorder caused by mutations in the dystrophin gene located on the short arm of the X chromosome. The absence of, or defects in, dystrophin results in chronic inflammation, progressive muscle degeneration, and replacement of muscle with fibroadipose tissues [77]. DMD patients often lose independent ambulation by the time they reach 13 years of age and generally die of respiratory failure in their late teens or early twenties [78]. Myotonic dystrophy type 1 (DM1) and type 2 (DM2) represent the most frequent multisystemic muscular dystrophies in adulthood [79]. DM1 and DM2 are rare disorders caused by noncoding intragenic repeat tract expansions of CTG (*DMPK* gene) and CCTG (*CNBP1* gene), which are pathogenic above 50 or 75 units, respectively [80, 81]. DM patients have primarily affected skeletal musculature and display muscle weakness (myopathy), muscle wasting (atrophy), and myotonia as the most recognized signs [79, 81]. DM1 and DM2 are characterized as multisystem progressive disorders, with the most frequent causes of death being respiratory failure and heart conduction defects.

Comprehensive miRNA expression profiling has revealed that miRNA dysregulation is a common feature in DMD and DM muscles. Nevertheless, the specific role that this dysregulation exerts over dystrophic muscle precursor cell biology is poorly understood. In this sense, Alexander et al. showed that miR-486 is downregulated in human DMD myoblast during myogenic differentiation as they are compared with wild type [50]. As we have mentioned before, this miR acts as a negative regulator of the PTEN/AKT signaling components and their downstream effector during skeletal muscle regeneration [50]. Lack of miR-486 PTEN/AKT signaling deregulation worsens myoblast differentiation and, consequently, could aggravate the DMD phenotype. Hence, modulation of the PTEN/AKT signaling pathway through miR-486 expression has the potential to be a therapy for treating DMD. Nevertheless, this hypothesis remains elusive. The same group has also showed that miR-199a is overexpressed in human DMD myoblast during myogenic differentiation as they are compared with wild type [65]. As we have mentioned before, miR-199a acts as a potential regulator of myogenesis through suppression of WNT signaling factors that act to balance myogenic cell proliferation and differentiation. Alexander et al. showed how muscle-specific overexpression of miR-199a transcript *in vivo* results in myofiber disruption and early lethality in zebrafish. However, in this work, the authors use a mylz2-promoter sequence to drive miR-199a-5p expression in skeletal muscle. This promoter is active specifically in zebrafish skeletal muscle fibers, excluding muscle stem cell progenitors [82], hence the effect of miR-199a overexpression in muscle precursor cells still remain unknown. Nevertheless, modulation of miR-199a also emerges with an important potential to be a therapy for treating DMD. In a more specific approach, de Arcangelis et al. showed that the expression level of miR-222 was 50% higher in SCs from dystrophic mdx muscles than in wild type cells. This leads to the decrease in β1-syntrophin expression by specifically binding to the 3′-UTR of β 1-syntrophin, a component of dystrophin-associated protein complex (DAPC), suggesting that the absence

of β1-syntrophin could worsen the disease [83]. Nevertheless, the authors did not explore downstream effects mediated by miR-222 overexpression in SCs, hence its impact in muscle precursor cells still remains elusive.

To obtain primary dystrophic muscle precursors cell cultures, either SCs or their derived myoblasts, is an extreme difficult task to achieve since their pathological backgrounds prevent their proper expansion *in vitro*. Some groups have tried to partially solve this problem by obtaining myogenic cell lines from dystrophic patients derived from immortalized fibroblasts by using retroviral-mediated expression of murine MyoD under the control of the Tet-on inducible construct or by transduction of the TERT and inducible Myod genes [84, 85]. By using this approach, Fernandez-Costa et al. showed that, as happened in DM1 Drosophila model muscle cells, myogenic cell lines derived from DM1 patients showed a downregulation of miR-1, miR-7, and miR-10, demonstrating the conservation of miRNA dysregulation triggered by expanded CTG repeats between the Drosophila model and humans [85]. Although overexpression of some of their putative targets was validated by RT-qPCR, the mechanisms by which this downregulation induces in DM1-myoblast maturation still remains unknown. Similarly, Cappella et al. showed a significant miRNA29c downregulation in human DM1 myotubes [84]. Since miRNA29c targets ankyrin repeat and SOCS box containing 2 (Asb2), a subunit of a multimeric E3 ubiquitin-ligase complex that negatively regulates muscle fiber mass [84], miRNA29c downregulation in DM1 could affect total muscle mass and worsen disease progression. In agreement with Cappella et al., Wang et al. (2012) have demonstrated that the loss of miR-29 impairs myogenic differentiation in mdx myoblasts [86]. This impairment may be due to the control exercised by miR29 in fibrosis.

In this regard, we must stress that miR-29-family miRNAs display a crucial role in the regulation of extracellular matrix genes and in fibrosis [87]. The replacement of muscle with fibroadipose tissues is a major pathological hallmark of DMD and DM [77, 84]. The canonical TGF-β/Smad signaling pathway, a well-known pathway involved in fibrosis formation, appears to negatively regulate the expression of miR-29, thereby promoting the conversion of myoblasts in myofibroblasts [86, 88]. During this transdifferentiation, activated TGF-β signaling induces Smad3 translocation into nucleus where it binds to miR-29 promoter, resulting in MyoD dissociation as well as YY1/Ezh2 stabilization. This causes a loss of miR-29 expression and increased expression of collagens and Lims1, leading to the transdifferentiation of myoblasts into myofibroblasts. All together, these data suggest that miR-29 could be an important molecular target for treating fibrosis associated to DMD and DM phenotypes.

Beyond the mere description, the works presented in this section provide us with very valuable information that can help us find new therapeutic targets on which to focus the development of drugs that would help us to alleviate the effects of dystrophic pathologies. We will discuss this issue in the next section.

5.2 miRNA as therapeutic targets in DMD and DM

As we have previously illustrated, several miRNAs are significantly dysregulated in DMD and DM muscular dystrophies and are able to modify muscle cell behavior in this context. For those downregulated, miRNA replacement can be conducted to restore its function by introducing a miRNA mimic product. The miR mimic technology utilizes synthetic, modified oligonucleotides that can bind to the unique sequence of target genes (mRNAs) in a gene-specific manner and elicit posttranscriptional repressive effects as an endogenous miRNA does [89]. Alternatively, application of miR mimics targeting the disease-causing genes to prevent their upregulation may be an efficient maneuver to tackle the problem [89]. For those miRNAs upregulated, inhibition can be conducted by using antimiR products.

Different types of antimiR products exist based on their mechanism of action. As happens for the miRNA mimic product, antimiRs comprise numerous classes of chemically modified oligonucleotides and nucleic acid analogs like locked nucleic acids (LNAs), 2′-O-methyl (2′-O-Me) oligos, 2′-O-methoxyethyl (2′-O-MOE) oligos, antagomiRs, peptide nucleic acids (PNAs), and phosphorodiamidate morpholinos (PMOs) [90]. These chemical modifications are implemented to provide resistance to cellular nucleases and to increase affinity toward complementary miRNA sequences [91, 92]. In addition, some antimiRs have flanking sequences or are connected to lipids through the use of linkers [93]. All the molecules that we have mentioned so far induce transient effects either because they are diluted by successive cell divisions or because they are metabolized in the cytoplasm [93]. To achieve long-term suppression of a specific miRNA, specialized plasmid and virus vectors carrying expression units for these inhibitory RNA molecules have also been developed [94]. In this regard, as an alternative to chemically modified antisense oligonucleotides, Ebert et al. developed miRNA inhibitors that can be expressed in cells as RNAs produced from transgenes [95]. Termed "miRNA sponges," these competitive inhibitors are transcripts expressed from strong promoters, containing multiple, tandem binding sites to an miRNA of interest. When vectors encoding these sponges are transfected into cultured cells, sponges derepress miRNA targets at least as strongly as chemically modified antisense oligonucleotides [95].

Muscular dystrophy	Molecular approach	Mimic/target miRNA	References
Duchenne muscular dystrophy	miRNA mimic/antimiR	miR-21	[97, 98]
	miRNA mimic	miR-29	[86, 97, 99]
	miRNA sponge	miR-31	[100]
	miRNA mimic/antimiR	miR-34c	[101]
	miRNA mimic/antimiR	miR-188	[102]
	miRNA sponge	miR-206	[103]
	miRNA mimic	miR-431	[104]
	miRNA mimic/antimiR	miR-675	[98]
	miRNA mimic/antimiR	miR-708	[101]
	miRNA mimic	miR-10	[85]
	miRNA sponge	miR-277	[105]
Myotonic dystrophy	miRNA sponge	miR-304	[105]
	antimiR	miR-23b	[106]
	antimiR	miR-218	[106]
	miRNA mimic	miR-1	[107, 108]
	miRNA mimic	miR-206	[107, 109]
	miRNA mimic	miR-148a	[107]
	miRNA mimic	miR-214	[107]
	miRNA mimic	miR-15b	[107]
	miRNA mimic	miR-16	[107]
	miRNA mimic	miR-30	[110]

Table 2.
miRs' therapeutic assays in animal models.

These miRNA sponge vectors inhibit miRNA function efficiently but for no longer than 1 month [96]. This problem has been partially solved by the development of "tough decoy RNAs" technology [93, 96]. Tough decoy inhibitor is a 60 base pair long hairpin-shaped inhibitor with a large internal bulge containing two miRNA recognition sites [93, 96]. Through plasmid- or lentivirus-based vectors, these molecules are efficiently exportable to the cytoplasm, where they target the highly potent miRNA inhibitory system which persists for well over 1 month [93, 96]. In the field of muscular dystrophies, many of these approaches have been tested *in vitro* and *in vivo* with animal models (**Table 2**). However, doubts related to the safety and efficiency of delivery still discourage the use of these molecules in humans.

6. Conclusions and perspectives

At present, a critical point for the development of effective strategies for treating muscle disorders is optimizing approaches to target muscle stem cells in order to increase the ability to regenerate lost tissue. In the context of muscle regeneration, emerging scientific evidence supports that *miRNAs* play a critical role in skeletal muscle, as they are required for the development and differentiation of this tissue. In addition, deregulation of *miRNAs* in muscle degenerative diseases suggests that gene-based therapies of *miRNAs* can be effective in treating muscle-related disorders. In this sense, restoration of non-pathological level of miRs expression would help to ameliorate these pathologies. Although many *in vitro* approaches have been accomplished in this regard, *in vivo* strategies remain poorly explored since the main shortcoming of the field lies in the ineffective delivery of either mimics or antimiR molecules. These molecules must overcome numerous roadblocks as canonical physiological pharmacokinetic and cellular uptake barriers as well as noncanonical barriers, such as intracellular miRNA localization and trafficking, off-target toxicities, and other intrinsic limitations. Improvement in this task will be the upcoming challenge for the next years by looking for strategies that allow us to aim these molecules in a specific fashion to muscle progenitor cells, thus minimizing the off-target effects of non-muscle tissues.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Francisco Hernandez-Torres and Amelia Aranega conceived of the structure and content. Francisco Hernandez-Torres wrote the first draft document. Francisco Hernandez-Torres, Lara Rodriguez-Outeiriño, and Lidia Matias-Valiente designed and produced the figures and tables. Estefania Lozano-Velasco and Diego Franco critically revised the manuscript for intellectual content. All authors provided content and writing feedback and reviewed the final manuscript. Amelia Aranega corrected, edited, and approved the final version of the document to be published.

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