Non coding RNAs and Duchenne Muscular Dystrophy

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Abstract

Purpose of review

Non coding RNAs (ncRNAs), such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) modulate gene transcription or translation in response to environmental stressors and other stimuli. A role for ncRNAs in muscle pathologies has been demonstrated and further evidence suggests that ncRNAs also play a role in Duchenne Muscular Dystrophy (DMD).

Key findings

Studies investigating the differential expression of miRNAs in biological fluids between DMD patients, and models of dystrophin deficiency (the *MDX* mouse model, canine models of DMD) and controls have been published, as have their role in fibrosis. *miRNA-1*, *-133a,b*, and *-206* are the most reported miRNAs, and have been implicated in myogenic differentiation, fibrosis, and regulation of utrophin and dystrophin translation. Overexpression of *miR-486* slows down disease progression in the *MDX* mouse model. lncRNAs, such as *hsa-lnc-31* and *linc-MD1*, are differentially expressed in DMD patients and may, in part, have a mechanism of action via targeting of miRNAs, such as *miR-133b*. Although many of these recent findings need to be confirmed, ncRNAs may prove to be useful as potential biomarkers of disease. However, their use as therapeutic targets in DMD remains unclear.

Summary

There may be a role for using circulating miRNAs as biomarkers of disease status. The use of ncRNAs as a therapeutic option for DMD remains to be determined.

Keywords

Duchenne Muscular Dystrophy; miRNA; lncRNA; *MDX* mice; *GRMD* dog

Introduction

Duchenne Muscular Dystrophy (DMD) is the most common muscular dystrophy affecting children. It is a severe X-linked neuromuscular disease caused by mutations in the dystrophin gene. DMD is characterized by a rapid progression of muscle degeneration that leads to the loss of ambulation and death within the second decade of life without medical intervention [1–3].

Dystrophin has a major structural role in muscle as it links the internal cytoskeleton to the extracellular matrix. The amino-terminus of dystrophin binds to F-actin and the carboxyl terminus to the dystrophin-associated protein complex (DAPC) at the sarcolemma [4]. The DAPC includes the dystroglycans, sarcoglycans, dystrobrevin and syntrophin, and mutations in any of these components cause autosomally inherited muscular dystrophies [3]. The DAPC is destabilized when dystrophin is absent, which results in diminished levels of its composite proteins [5]. This, in turn, leads to progressive fibre damage and membrane leakage. Furthermore, the DAPC has a signalling role, the loss of which also contributes to pathogenesis [4]. DMD patients are usually wheelchair-bound by the age of 12 and die of respiratory failure in their late teens or, with the help of respiratory support, in the 3rd or 4th decade of life. Cardiac involvement is invariable, indicating that any therapeutic agent must also target the cardiac muscle.

Noncoding RNAs (ncRNAs) have emerged as novel molecules that may be important in DMD [6]. ncRNAs can be sub-classified into three groups: housekeeping RNAs (ribosomal, transfer and splicesomal), long noncoding (pseudogenes, intronic and intergenic), and the small ncRNAs (piwi-associated RNA, endogenous short interfering RNA (siRNA) and microRNAs (miRNAs)). Of these, the miRNAs are the most studied in DMD. miRNAs are small RNAs, consisting of ~22 nts that are highly conserved across species and act as regulators of both genes and gene networks [7]. They are able to induce messenger RNA

(mRNA) degradation and/or inhibit mRNA translation, and as many as 60 % of mRNAs may be targets for miRNAs [8]. miRNAs control the signalling pathways in most cell types, have a role in development and cellular phenotype and regulate myogenic proliferation and fibrosis. Hence, miRNAs have been proposed to have a pathophysiological role in DMD. Furthermore, because miRNAs have been found to be extremely stable in serum, they may also be used as biomarkers to aid in the DMD diagnosis, as well as monitoring disease progression, and response to therapy. This review will focus on the association between miRNAs and DMD by reviewing the current knowledge (**Table 1**), and also reflect upon the lesser known, but also important lncRNAs.

Human

Eisenberg *et al* (2007) described 185 miRNAs that are up- or down-regulated in 10 major muscular disorders in humans (DMD, the milder allelic variant Becker muscular dystrophy, facioscapulohumeral muscular dystrophy, limb-girdle muscular dystrophies types 2A and 2B, Miyoshi myopathy, nemaline myopathy, polymyositis, dermatomyositis, and inclusion body myositis). Although five miRNAs were found to be consistently dysregulated in almost all muscle specimens analysed, pointing to possible involvement of a common regulatory mechanism, others were dysregulated only in one disease and not at all in the other disorders [9]. 29 miRNAs were increased in expression in DMD when compared to control patients (*miR-21, -34a, -130a, -146b, -148, -154, -155, -199a, -199b, -210, -214, -221, -222, -299, -335, -368, -376a, -379, -381, -432, -452, -487b, -495,-2537, -4983, -13145,* and *-13258*), and 2 significantly decreased in expression (*miR-30a* and *-11040*) [9]. Zaharieva *et al* (2013) furthered these studies, and demonstrated that *miR-1*, *-133a,b* and *-206* were also upregulated in DMD, and that patients with low forced vital capacity (FVC) values, indicating respiratory muscle weakness, present with lower levels of serum *miR-1* and *-133b* [10]. Further similar

studies [11–14], have suggested a panel of miRNAs including *miR-1, -31, -133, -206, -208a,-208b*, and *-499*, that may be useful as biomarkers to diagnose patients, as well as profiling of different muscle cell phenotypes, including cardiac muscles, skeletal muscles, and vascular and visceral smooth muscles (*miR-1, -133a, -145,-206, -208a, -208b, -499*) [15]. Of course, ensuring that a standard SOP is followed by all such studies is vital to ensure the detection of "true" biomarkers. As such, the gold standard for miRNA detection methodology is RT-qPCR and to normalize to a synthetic spike-in control oligonucleotide [16].

As well as acting as possible disease biomarkers, the targets for some of these miRNAs have also been described. For example, *miR-21* and *miR-29* play opposing roles in DMD muscle fibrosis, likely by targeting Collagen, Type III, Alpha 1 (*COL3A1*), Fibrillin 1 (*FBN1*) and YY1 Transcription Factor (*YY1*), either directly or indirectly [17]. Regulation of *miR-199a-5p* in a serum response factor (SRF)-dependent manner in human primary myoblasts and myotubes results in changes in cellular size, proliferation, and differentiation, via targeting of several myogenic cell proliferation and differentiation regulatory factors within the WNT signalling pathway, including Frizzled Class Receptor 4 (*FZD4*), Jagged 1 (*JAG1*), and Wingless-Type MMTV Integration Site Family Member 2 (*WNT2*) [18]. Differential Histone Deacetylase 2 (*HDAC2*) nitrosylation, observed in DMD when compared to non-disease controls deregulates *miR-1, -29*, and *-206*, which are linked to the G6PD enzyme, to extracellular proteins and the fibrotic process, and to muscle regeneration through repression of the satellite cell specific factor, Paired Box 7 (*Pax7*), in activated satellite cells [19]. Furthermore, Greco *et al* (2009) have recently identified miRNAs involved in the pathological pathways activated in skeletal muscle damage and regeneration triggered by a lack of dystrophin [20]. These DMD-signature miRNAs are divided into 3 classes. 1) Regeneration miRNAs (*miR-31, -34c, -206, -335, -449,* and *-494*) induced in DMD patients. 2) Degenerative-miRNAs (*miR-1, -29c*, and *-135a*) down-modulated in DMD patients and

linked to myofiber loss and fibrosis. 3) Inflammatory miRNAs (*miR-222* and *-223*), whose expression correlated with the presence of infiltrating inflammatory cells [20]. Inhibition of *miR-486* in normal muscle myoblasts results in inhibited migration and failure to repair a wound *in-vitro*, and its overexpression results in increased proliferation, by regulating the phosphatase and tensin homolog deleted on chromosome 10/AKT (PTEN/AKT) pathway [21].

Interestingly, miRNAs may also be beneficial in improving exon skipping therapy in DMD patients. Cazzella *et al* (2012), has shown that the selection of U1 snRNA-antisense constructs to confer effective rescue of dystrophin synthesis in a Δ44 DMD genetic background, through skipping of exon 45. The restored dystrophin is able to recover the delay in myogenic marker expression in differentiating myoblasts, relocalise neuronal nitric oxide synthase (*nNOS*) and to rescue expression of miRNAs (including *miR-1* and *-29c*) previously shown to be sensitive to the Dystrophin-nNOS-HDAC2 pathway [22]. Furthermore, *miR-31* represses dystrophin expression by targeting its 3' untranslated region, and in human DMD myoblasts treated with antisense oligonucleotides to induce exon skipping, *-miR-31* inhibition increases dystrophin restoration, suggesting that modulating *miR-31* expression may provide an additional strategy for those DMD therapies that are aimed at efficiently recovering dystrophin synthesis [23].

Other muscle specific miRNAs that are known to control both inflammation and proliferation in Airway Smooth Muscle (ASM), such as *miR-145* [24], *miR-150, -371-5p, -718, -940, -1181, -1207-5p, -1915, -3663-3p* [25], and *miR-221* [26], may also prove important in DMD, but have yet to be studied.

MDX mouse

There is a certain degree of overlap between miRNAs in human DMD and 'naturally' occurring animal models, suggesting evolutionary conservation of miRNA expression during DMD development. Indeed, Greco *et al* (2009) not only described the 3 classes of signature miRNAs (above), in human DMD, but also in *MDX* mice [20]. Furthermore, *miR-1, -21, -29, -31, -133a, -148, -206, -222,* and *-335* are expressed in both species; however *miR-29, -21* and *-148* are differentially expressed [27–36]. *miR-23a, -30e, -34c, -193b, -223, -434, -449,* and *-494*, as yet, remain unique to the *MDX* mice. The distribution of miRNAs in mouse muscle is better understood, compared to the human counterpart, with distinct patterns having been observed in the tibialis anterior (TA) muscle [31], diaphragm [37], heart [38], and the soleus and plantaris muscles in the leg [37]. *miR-206* is particularly important in *MDX* mice, as it has been shown to be induced by fibro-adipogenic progenitors (FAPs), which are known to contribute to the pathogenesis and progression of DMD [39], as well as being modulated by mouse insulin-like growth factors (mIGF-1) [40]. The activity of *miR-206* included both skeletal muscle regeneration [41], and differentiation of satellite cells via TNF Receptor-Associated Factor 6, E3 Ubiquitin Protein Ligase (*TRAF6*) regulation [42]. miRNA targets are also better defined in the *MDX* model, with roles for the inhibition of dystrophin (*miR-31, -146b* and *-374*) [43] in Becker muscular dystrophy, improvement of disease progression via targeting of Dedicator Of Cytokinesis 3 (*DOCK3*) by *miR-486* [44], downregulation of peroxisome proliferator-activated receptor γ1 (*Pparγ1*) by *miR-27b* [45], promotion of myogenic differentiation via *miR-1, -133* and *-102* targeting of *BAF60* [46], and promotion of myofibroblast differentiation through the action of *miR-29* targeting of Microfibrillar Associated Protein 5 (*Mfap5*) [47]. Recently, Israeli *et al* (2016), have reported upon the role of the above miRNAs (including *miR-1*, *-21*, *-31*, *-133*, *-142-3p*, *-149-5p*, *-193b*, *-206*, and *-378a-3p*) for the evaluation of therapeutic outcome in medical approaches for numerous muscular dystrophies, including DMD [48].

MDX mice are not the only mouse lineage to prove useful in our understanding of DMD. Indeed, the fact that extraocular muscles (EOM) are "spared" in advanced DMD [49], led to Zeiger and Khurana (2010), profiling the miRNA signature of EOM in WT mice, and discovering that *miR-1, -133a* and *-133b* are decreased in expression, and *miR-206*, is increased in expression, possibly explaining the differential sensitivity of this muscle allotype to dystrophin-deficiency [50]. Additionally, Ghahramani *et al*, (2010) preferred to knock-out dystrophin with RNAi in C57BL10 mice and study the transcriptome [51]. This approach, not only highlighted a change in expression of known miRNAs such as *miR-208b*, but also identified novel miRNAs including *miR-128, -684* and *-1192* [51]. Furthermore, mouse cell lines (i.e. C2C12) have proven useful in the study of DMD, to describe the posttranscriptional regulation of utrophin via miRNA targeting (*let-7c, miR-133b, -150, -96b, -206,* and *-296*) [52,53].

Canine and Ovine In-vivo Models

Unlike the *MDX* mouse, which remains relatively normal (clinically), affected canine models of DMD develop progressive, fatal disease strikingly similar to the human condition. Accordingly, studies in the canine dystrophin-deficient models, such as golden retriever muscular dystrophy (*GRMD*) and canine X-linked muscular dystrophy in Japan dog model (*CXMD(J)*) may be more likely than those in *MDX* mice to predict pathogenesis and outcome of treatment in DMD. As yet, however, microRNA studies in these models are limited. Both *miR-1* and *-133a* have been shown to be decreased in *GRMD* [54], and 9 miRNAs have been proposed to act as serum biomarkers (*miR-1, -95, -133, -206, -208a, -208b, -378, -499,* and *-539*) [55]. Of these, two miRNAs (*miR-208b* and *-539*), have been shown to contribute to hypertrophy and the functional sparing of the cranial sartorius (SC) muscle [56]. Additional

studies in the Japanese CXMD(J), further highlight the importance of the microRNAs; *miR-1, -133a* and -*206* [29].

Interestingly, two of the most frequently reported muscle miRNAs; *miR-1* and -*206*, are proposed to target the 3'-UTR of the myostatin gene in the Texel sheep leading to inhibition of myostatin expression, which likely causes the muscular hypertrophy phenotype of this breed of sheep [57].

lncRNAs

In addition to the miRNA family of short noncoding RNAs (< 200 nucleotides), there is now accumulating evidence that long noncoding RNAs (lncRNAs) with more than 200 nucleotides can regulate multiple biological responses and that changes in their expression may be related to the development of disease [58,59]. For example, primary human airway smooth muscle (ASM) cell phenotype might, in part, be mediated through alterations in lncRNA expression [25], and targeting of the lncRNA, *PVT1*, has been demonstrated to control both the aberrant proliferation and inflammatory mediator release from ASM cells isolated from patients with severe asthma [60]. Although studies on lncRNAs in DMD are limited, a handful of papers are starting to highlight the possible importance of these novel RNAs.

For example, Ballarino *et al*, (2015) utilized a transcriptomic approach to identify novel lncRNAs in murine myoblast differentiation [61]. Furthermore, they demonstrated that *lnc-31* and its human homologue *hsa-lnc-31* are expressed in proliferating myoblasts, where they counteract differentiation. This is not the only lncRNA to be commonly expressed in both mouse models and in humans, but *linc-MD1* has been shown to be expressed during early stages of normal murine myoblast differentiation as well as human primary myoblasts from DMD patients [62], additionally its mechanism of action as a 'sponge' for *miR-133b* was

described by Twayana *et al*, (2013) [63]. Clearly, further studies are needed to delineate the role of these novel transcripts.

Conclusion

Recent studies indicate that ncRNAs may be important in diagnosing DMD, and in various aspects of its pathogenesis. However, although treatment of DMD, and other neuromuscular diseases currently involves oligonucleotide targeting (extensively reviewed in [64,65]), targeting of ncRNAs, or indeed, the effect of such therapies upon important ncRNAs remains to be seen. miRNAs (and to a lesser extent, currently, lncRNAs) appear to be important in all areas of DMD, and there is a potential for ncRNA research to uncover as yet unknown mechanisms in the pathogenesis DMD as well as being developed into novel therapies.

microRNA	Function	Target
Human		
miR-21, miR-34a, miR-130a, miR-146b, miR-148, miR-154, miR-155, miR-199a, miR-199b, miR-210, miR-214, miR-221, miR-222, miR-299, miR-335, miR-368, miR-376a, miR-379, miR-381, miR-432, miR-452, miR-487b, miR-495, miR-2537, miR-4983, miR-13145, miR-13258	Increased in DMD muscle [9]	?
miR-30a, miR-11040	Decreased in DMD muscle [9]	?
miR-1, miR-133b	Decreased in DMD patients that had low FVC [10]	?
miR-1, miR-31, miR-133, miR-206, miR-208a, miR-208b, miR-499	Serum biomarker in patients [10–14]	?
miR-1, miR-133a, miR-145, miR-206, miR-208a, miR-208b, miR-499	Profiling of muscle cells [15]	?
miR-21, miR-29	Reduced in DMD muscle and myoblasts [17]	Likely: COL3A1, FBN1 and YY1
miR-199a	Dysregulated in dystrophin-deficient zebrafish, *MDX* mice, and DMD human muscle biopsies [18]	FZD4, JAG1, and WNT2
miR-1, miR-29, miR-206	Deregulated by HDAC2 nitrosylation [19]	Pax7
miR-1, miR-29, miR-135a	Linked to myofiber loss and fibrosis [20]	?
miR-1, miR-29	Recovery through exon 45 skipping [22]	?
miR-486	Regulates PTEN/AKT pathway in dystrophin-deficient muscle [21]	PTEN/AKT pathway
miR-31	Represses dystrophin expression at 3'-end [23]	Dystrophin
MDX Mice		
miR-1, miR-23a, miR-29, miR-30e, miR-31, miR-34c, miR-133a, miR-193b, miR-206, miR-222, miR-223, miR-335, miR-434, miR-449, miR-494	Increased in *MDX* mice [20,27–34]	HDAC2, β1-syntrophin
miR-21, miR-143, miR-146a, miR-148, miR-429, miR-451	Decreased in *MDX* mice [34–36]	?
miR-18a, miR-21, miR-34b, miR-146b, miR-501, miR-675, miR-1983	Increased in *MDX* mice TA muscle [31]	?
miR-29c, miR-101b, miR-143, miR-181a, miR-329, miR-337, miR-381, miR-434, miR-539	Decreased in *MDX* mice TA muscle [31]	?
miR-206	Increased in *MDX* mouse diaphragm [37]	?
miR-448	Decreased in the hearts of *MDX* mice [38]	Ncf1
miR-133a	Decreased in *MDX* mouse soleus and plantaris [37]	?
miR-1, miR-133a, miR-145, miR-206, miR-208a, miR-208b, miR-499	Profiling of muscle cells [15]	?
miR-199a	Dysregulated in dystrophin-deficient zebrafish, *MDX* mice, and human muscle biopsies [18]	FZD4, JAG1, and

		WNT2
miR-206	Induced by FAPs [39]	?
miR-31, miR-146b, miR-374	Inhibits dystrophin [43]	Dystrophin
miR-486	Overexpression halts disease progression [44]	DOCK3
miR-1, miR-29, miR-135a	Linked to myofiber loss and fibrosis [20]	?
miR-24, miR-206	Modulated by mIGF-1 expression [40]	?
miR-27b	NO increased expression leading to downregulation of Pparγ1 expression [45]	Pparγ1
miR-1, miR-206	Promote differentiation of satellite cells [42]	?
miR-1, miR-133, miR-102	Promotes myogenic differentiation [46]	BAF60A, BAF60B
miR-206	Promotes skeletal muscle regeneration [41]	?
miR-21, miR-29	Reduced in DMD muscle and myoblasts [17]	Likely: COL3A1, FBN1 and YY1
miR-29	Regulated by TGF-β, and promotes myofibroblast differentiation [47]	Mfap5
miR-486	Regulates PTEN/AKT pathway in dystrophin-deficient muscle [21]	PTEN/AKT pathway
WT Mice		
miR-206	Increased in EOM [50]	?
miR-1, miR-133a, miR-133b	Decreased in EOM [50]	?
C57BL10 mice		
miR-128, miR-208b, miR-684, miR-1192	Dysregulated by dystrophin deficiency [51]	?
C2C12 Mouse cell line		
miR-206, let-7c, miR-133b, miR-150, miR-196b, miR-296	Posttranscriptional regulation of utrophin [52,53]	Utrophin
GRMD		
miR-1, miR-133a	Decreased in GRMD [54]	?
miR-1, miR-95, miR-133, miR-206, miR-208a, miR-208b, miR-378, miR-499, miR-539	Serum biomarker in GRMD [55]	?
miR-208b, miR-539	Contributes to hypertrophy and functional sparing of the CS [56]	?
CXMD(J)		
miR-1, miR-133a, miR-206	Increased in CXMD(J) [29]	?
Texel Sheep		
miR-1, miR-206	Causes muscular hypertrophy in Texel sheep [57]	Myostatin

Table 1. microRNAs and DMD

Definition of abbreviations: FVC, forced vital capacity; COL3A1, Collagen, Type III, Alpha 1; FBN1, Fibrillin 1; YY1, YY1 Transcription Factor; *MDX*, X chromosome-linked muscular dystrophy; FZD4, Frizzled Class Receptor 4; JAG1, Jagged 1; WNT2, Wingless-Type MMTV Integration Site Family Member 2; Pax7, Paired Box 7; HDAC2, Histone Deacetylase 2; PTEN, Phosphatase And Tensin Homolog; AKT, V-Akt Murine Thymoma Viral Oncogene Homolog; TA, Tibialis anterior; Ncf1, Neutrophil Cytosolic Factor 1; FAP,

Fibro-adipogenic progenitor; DOCK3, Dedicator Of Cytokinesis 3; mIFG1, mouse Insulin-Like Growth Factor 1; NO, nitric oxide; Pparγ1, Peroxisome proliferator-activated receptor γ1; TGF, Transforming growth factor; EOM, extraocular muscle; GRMD, Golden retriever muscular dystrophy; CXMD(J), canine X-linked muscular dystrophy in Japan.

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