



Non coding RNAs and Duchenne Muscular Dystrophy

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1 **Non coding RNAs and Duchenne Muscular Dystrophy**

2

3 ***Abstract***

4 ***Purpose of review***

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

10 ***Key findings***

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

22 ***Summary***

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

25

26 **Keywords**

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

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28 *Introduction*

29 Duchenne Muscular Dystrophy (DMD) is the most common muscular dystrophy affecting
30 children. It is a severe X-linked neuromuscular disease caused by mutations in the dystrophin
31 gene. DMD is characterized by a rapid progression of muscle degeneration that leads to the
32 loss of ambulation and death within the second decade of life without medical intervention
33 [1–3].

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

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

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

62

63 **Human**

64 Eisenberg *et al* (2007) described 185 miRNAs that are up- or down-regulated in 10 major
65 muscular disorders in humans (DMD, the milder allelic variant Becker muscular dystrophy,
66 facioscapulohumeral muscular dystrophy, limb-girdle muscular dystrophies types 2A and 2B,
67 Miyoshi myopathy, nemaline myopathy, polymyositis, dermatomyositis, and inclusion body
68 myositis). Although five miRNAs were found to be consistently dysregulated in almost all
69 muscle specimens analysed, pointing to possible involvement of a common regulatory
70 mechanism, others were dysregulated only in one disease and not at all in the other disorders
71 [9]. 29 miRNAs were increased in expression in DMD when compared to control patients
72 (*miR-21*, *-34a*, *-130a*, *-146b*, *-148*, *-154*, *-155*, *-199a*, *-199b*, *-210*, *-214*, *-221*, *-222*, *-299*, *-*
73 *335*, *-368*, *-376a*, *-379*, *-381*, *-432*, *-452*, *-487b*, *-495*, *-2537*, *-4983*, *-13145*, and *-13258*), and
74 2 significantly decreased in expression (*miR-30a* and *-11040*) [9]. Zaharieva *et al* (2013)
75 furthered these studies, and demonstrated that *miR-1*, *-133a,b* and *-206* were also upregulated
76 in DMD, and that patients with low forced vital capacity (FVC) values, indicating respiratory
77 muscle weakness, present with lower levels of serum *miR-1* and *-133b* [10]. Further similar

78 studies [11–14], have suggested a panel of miRNAs including *miR-1*, *-31*, *-133*, *-206*, *-208a*,
79 *208b*, and *-499*, that may be useful as biomarkers to diagnose patients, as well as profiling of
80 different muscle cell phenotypes, including cardiac muscles, skeletal muscles, and vascular
81 and visceral smooth muscles (*miR-1*, *-133a*, *-145*, *-206*, *-208a*, *-208b*, *-499*) [15]. Of course,
82 ensuring that a standard SOP is followed by all such studies is vital to ensure the detection of
83 “true” biomarkers. As such, the gold standard for miRNA detection methodology is RT-
84 qPCR and to normalize to a synthetic spike-in control oligonucleotide [16].

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

103 linked to myofiber loss and fibrosis. 3) Inflammatory miRNAs (*miR-222* and *-223*), whose
104 expression correlated with the presence of infiltrating inflammatory cells [20]. Inhibition of
105 *miR-486* in normal muscle myoblasts results in inhibited migration and failure to repair a
106 wound *in-vitro*, and its overexpression results in increased proliferation, by regulating the
107 phosphatase and tensin homolog deleted on chromosome 10/AKT (PTEN/AKT) pathway
108 [21].

109 Interestingly, miRNAs may also be beneficial in improving exon skipping therapy in DMD
110 patients. Cazzella *et al* (2012), has shown that the selection of U1 snRNA-antisense
111 constructs to confer effective rescue of dystrophin synthesis in a $\Delta 44$ DMD genetic
112 background, through skipping of exon 45. The restored dystrophin is able to recover the delay
113 in myogenic marker expression in differentiating myoblasts, relocalise neuronal nitric oxide
114 synthase (*nNOS*) and to rescue expression of miRNAs (including *miR-1* and *-29c*) previously
115 shown to be sensitive to the Dystrophin-nNOS-HDAC2 pathway [22]. Furthermore, *miR-31*
116 represses dystrophin expression by targeting its 3' untranslated region, and in human DMD
117 myoblasts treated with antisense oligonucleotides to induce exon skipping, *-miR-31* inhibition
118 increases dystrophin restoration, suggesting that modulating *miR-31* expression may provide
119 an additional strategy for those DMD therapies that are aimed at efficiently recovering
120 dystrophin synthesis [23].

121 Other muscle specific miRNAs that are known to control both inflammation and proliferation
122 in Airway Smooth Muscle (ASM), such as *miR-145* [24], *miR-150*, *-371-5p*, *-718*, *-940*, *-*
123 *1181*, *-1207-5p*, *-1915*, *-3663-3p* [25], and *miR-221* [26], may also prove important in DMD,
124 but have yet to be studied.

125

126 ***MDX mouse***

127 There is a certain degree of overlap between miRNAs in human DMD and ‘naturally’
128 occurring animal models, suggesting evolutionary conservation of miRNA expression during
129 DMD development. Indeed, Greco *et al* (2009) not only described the 3 classes of signature
130 miRNAs (above), in human DMD, but also in *MDX* mice [20]. Furthermore, *miR-1*, *-21*, *-29*,
131 *-31*, *-133a*, *-148*, *-206*, *-222*, and *-335* are expressed in both species; however *miR-29*, *-21*
132 and *-148* are differentially expressed [27–36]. *miR-23a*, *-30e*, *-34c*, *-193b*, *-223*, *-434*, *-449*,
133 and *-494*, as yet, remain unique to the *MDX* mice. The distribution of miRNAs in mouse
134 muscle is better understood, compared to the human counterpart, with distinct patterns having
135 been observed in the tibialis anterior (TA) muscle [31], diaphragm [37], heart [38], and the
136 soleus and plantaris muscles in the leg [37]. *miR-206* is particularly important in *MDX* mice,
137 as it has been shown to be induced by fibro-adipogenic progenitors (FAPs), which are known
138 to contribute to the pathogenesis and progression of DMD [39], as well as being modulated
139 by mouse insulin-like growth factors (mIGF-1) [40]. The activity of *miR-206* included both
140 skeletal muscle regeneration [41], and differentiation of satellite cells via TNF Receptor-
141 Associated Factor 6, E3 Ubiquitin Protein Ligase (*TRAF6*) regulation [42]. miRNA targets
142 are also better defined in the *MDX* model, with roles for the inhibition of dystrophin (*miR-31*,
143 *-146b* and *-374*) [43] in Becker muscular dystrophy, improvement of disease progression via
144 targeting of Deducator Of Cytokinesis 3 (*DOCK3*) by *miR-486* [44], downregulation of
145 peroxisome proliferator-activated receptor γ 1 (*Ppar γ 1*) by *miR-27b* [45], promotion of
146 myogenic differentiation via *miR-1*, *-133* and *-102* targeting of *BAF60* [46], and promotion of
147 myofibroblast differentiation through the action of *miR-29* targeting of Microfibrillar
148 Associated Protein 5 (*Mfap5*) [47]. Recently, Israeli *et al* (2016), have reported upon the role
149 of the above miRNAs (including *miR-1*, *-21*, *-31*, *-133*, *-142-3p*, *-149-5p*, *-193b*, *-206*, and -
150 *378a-3p*) for the evaluation of therapeutic outcome in medical approaches for numerous
151 muscular dystrophies, including DMD [48].

152 *MDX* mice are not the only mouse lineage to prove useful in our understanding of DMD.
153 Indeed, the fact that extraocular muscles (EOM) are “spared” in advanced DMD [49], led to
154 Zeiger and Khurana (2010), profiling the miRNA signature of EOM in WT mice, and
155 discovering that *miR-1*, *-133a* and *-133b* are decreased in expression, and *miR-206*, is
156 increased in expression, possibly explaining the differential sensitivity of this muscle allotype
157 to dystrophin-deficiency [50]. Additionally, Ghahramani *et al*, (2010) preferred to knock-out
158 dystrophin with RNAi in C57BL10 mice and study the transcriptome [51]. This approach, not
159 only highlighted a change in expression of known miRNAs such as *miR-208b*, but also
160 identified novel miRNAs including *miR-128*, *-684* and *-1192* [51]. Furthermore, mouse cell
161 lines (i.e. C2C12) have proven useful in the study of DMD, to describe the posttranscriptional
162 regulation of utrophin via miRNA targeting (*let-7c*, *miR-133b*, *-150*, *-96b*, *-206*, and *-296*)
163 [52,53].

164

165 ***Canine and Ovine In-vivo Models***

166 Unlike the *MDX* mouse, which remains relatively normal (clinically), affected canine models
167 of DMD develop progressive, fatal disease strikingly similar to the human condition.
168 Accordingly, studies in the canine dystrophin-deficient models, such as golden retriever
169 muscular dystrophy (*GRMD*) and canine X-linked muscular dystrophy in Japan dog model
170 (*CXMD(J)*) may be more likely than those in *MDX* mice to predict pathogenesis and outcome
171 of treatment in DMD. As yet, however, microRNA studies in these models are limited. Both
172 *miR-1* and *-133a* have been shown to be decreased in *GRMD* [54], and 9 miRNAs have been
173 proposed to act as serum biomarkers (*miR-1*, *-95*, *-133*, *-206*, *-208a*, *-208b*, *-378*, *-499*, and *-*
174 *539*) [55]. Of these, two miRNAs (*miR-208b* and *-539*), have been shown to contribute to
175 hypertrophy and the functional sparing of the cranial sartorius (SC) muscle [56]. Additional

176 studies in the Japanese CXMD(J), further highlight the importance of the microRNAs; *miR-1*,
177 *-133a* and *-206* [29].

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

182

183 *lncRNAs*

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

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

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

203

204 ***Conclusion***

205 Recent studies indicate that ncRNAs may be important in diagnosing DMD, and in various
206 aspects of its pathogenesis. However, although treatment of DMD, and other neuromuscular
207 diseases currently involves oligonucleotide targeting (extensively reviewed in [64,65]),
208 targeting of ncRNAs, or indeed, the effect of such therapies upon important ncRNAs remains
209 to be seen. miRNAs (and to a lesser extent, currently, lncRNAs) appear to be important in all
210 areas of DMD, and there is a potential for ncRNA research to uncover as yet unknown
211 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, <i>MDX</i> 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 <i>MDX</i> mice [20,27–34]	HDAC2, β 1-syntrophin
miR-21, miR-143, miR-146a, miR-148, miR-429, miR-451	Decreased in <i>MDX</i> mice [34–36]	?
miR-18a, miR-21, miR-34b, miR-146b, miR-501, miR-675, miR-1983	Increased in <i>MDX</i> mice TA muscle [31]	?
miR-29c, miR-101b, miR-143, miR-181a, miR-329, miR-337, miR-381, miR-434, miR-539	Decreased in <i>MDX</i> mice TA muscle [31]	?
miR-206	Increased in <i>MDX</i> mouse diaphragm [37]	?
miR-448	Decreased in the hearts of <i>MDX</i> mice [38]	Ncf1
miR-133a	Decreased in <i>MDX</i> 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, <i>MDX</i> 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]
	miR-486	Overexpression halts disease progression [44]
	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 Ppar γ 1 expression [45]
	miR-1, miR-206	Promote differentiation of satellite cells [42]
	miR-1, miR-133, miR-102	Promotes myogenic differentiation [46]
	miR-206	Promotes skeletal muscle regeneration [41]
	miR-21, miR-29	Reduced in DMD muscle and myoblasts [17]
	miR-29	Regulated by TGF- β , and promotes myofibroblast differentiation [47]
	miR-486	Regulates PTEN/AKT pathway in dystrophin-deficient muscle [21]
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]
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]

212 **Table 1. microRNAs and DMD**

213 **Definition of abbreviations:** FVC, forced vital capacity; COL3A1, Collagen, Type III, Alpha
 214 1; FBN1, Fibrillin 1; YY1, YY1 Transcription Factor; *MDX*, X chromosome-linked muscular
 215 dystrophy; FZD4, Frizzled Class Receptor 4; JAG1, Jagged 1; WNT2, Wingless-Type
 216 MMTV Integration Site Family Member 2; Pax7, Paired Box 7; HDAC2, Histone
 217 Deacetylase 2; PTEN, Phosphatase And Tensin Homolog; AKT, V-Akt Murine Thymoma
 218 Viral Oncogene Homolog; TA, Tibialis anterior; Ncf1, Neutrophil Cytosolic Factor 1; FAP,

219 Fibro-adipogenic progenitor; DOCK3, Deducator Of Cytokines 3; mIFG1, mouse Insulin-
220 Like Growth Factor 1; NO, nitric oxide; Pparg1, Peroxisome proliferator-activated receptor
221 γ 1; TGF, Transforming growth factor; EOM, extraocular muscle; GRMD, Golden retriever
222 muscular dystrophy; CXMD(J), canine X-linked muscular dystrophy in Japan.

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224

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