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The role of non-coding RNAs in the pathogenesis of myotonic dystrophy type 1

El papel de los RNAs no-codificantes en la patogénesis de la distrofia miotónica tipo 1

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Abstract

Myotonic dystrophy type 1 (DM1) is the most common muscular dystrophy in adults with a prevalence of 1/8,000 worldwide. DM1 is a multisystem disorder with a complex pathophysiology. Spliciopathy is the mechanism with the greatest impact on the pathogenesis and is also the most studied. However, other mechanisms like deregulation of non-coding RNAs (ncRNAs) have been described that contribute to the pathogenesis. ncRNAs, particularly miRNAs, participate in the development, differentiation, and regeneration of muscle tissue in DM1. The potential role of some miRNAs as DM1 biomarkers has been revealed from patient's serum studies. More recent studies, described antisense DM1 RNA, now classified as a lncRNA, with a potential role in the formation of siRNAs, chromatin modifying, and RAN translation mechanisms. Nonetheless, lncRNA have not been described in DM1, and it would therefore be interesting to investigate the role they play in this disease. It appears that ncRNAs play an important role in DM1, adding new elements to the previously described mechanisms, which improve our understanding of this complex disease, leaving still a lot to be discovered.

Resumen

La distrofia miotónica tipo 1 (DM1) es la distrofia muscular más común en adultos con una prevalencia de 1/8,000 a nivel mundial. La DM1 es un trastorno multisistémico con una patofisiología compleja. El procesamiento alternativo es el mecanismo con el mayor impacto en la patogénesis y el más estudiado actualmente. Sin embargo, se ha descrito que otros mecanismos como desregulación de RNAs no-codificantes (ncRNAs) contribuyen a la patogénesis. Los ncRNAs, particularmente miRNAs, participan en el desarrollo, diferenciación y regeneración del tejido muscular en DM1. El potencial papel de algunos miRNAs como biomarcadores de DM1 ha sido revelado a partir de estudios con suero de pacientes. Estudios más recientes describieron la presencia de RNA antisentido, ahora clasificados como lncRNA, con un potencial papel en la formación de siRNAs, modificador de la cromatina y mecanismos de traducción RAN. No obstante, lncRNAs no han sido descritos en DM1 y, por lo tanto, podría ser interesante la investigación del papel que juegan en esta enfermedad. Parece que ncRNAs juegan un papel importante en DM1, adicionando nuevos elementos a los mecanismos descritos previamente, lo cual mejora nuestro entendimiento de esta enfermedad compleja, dejando mucho aún por descubrir.

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INTRODUCTION

Expansions of unstable microsatellite sequence, notably trinucleotide repeats, were identified as a novel mutational mechanism underlying more than 30 human disorders, with neurological and neuromuscular symptoms, including myotonic dystrophy type 1 (DM1).[1,2]

DM1 is the most common muscular dystrophy in adults, with a prevalence of 1/8,000 worldwide,[3] inherited in an autosomal dominant pattern. It is caused by the expanded CTG repeats in the 3' untranslated region (3'UTR) of the dystrophia myotonica protein kinase gene (DMPK) located on chromosome 19q 13.3.

DM1 is a multisystem disorder with a complex pathophysiology;[4] the symptoms and clinical findings include myotonia, muscle wasting, cardiac conduction defects, central nervous system alterations, cataracts, and insulin resistance, among others, whereas in the congenital form of DM1, cognitive dysfunction and mental retardation have also been documented.[5]

The number of CTG repeats ranges between five and thirty-five in the normal population and increases between fifty and several thousand in DM1 patients.[5,6] At the molecular level, mutant RNA with expanded CTG repeats is retained in nuclear aggregates that sequester proteins such as muscleblind-like proteins (MBNL1, MBNL2 and MBNL3), and alter the function of specificity protein 1 (SP1) and retinoic acid receptor gamma (RARg), resulting in alternative splicing and transcription deregulation.[7-9] Spliciopathy is the mechanism with the greatest impact on the pathogenesis and is also the most studied. However, other mechanisms that contribute to the pathogenesis such as changes in gene expression, translation efficiency, misregulated alternative polyadenylation and deregulation of non-coding RNAs (ncRNAs) have been described.[10-24]

Regarding ncRNAs, it has been shown that they are critical in regulatory activity in normal cellular development, function, and pathogenesis. They have recently been described as having an important role in neurodegenerative disorders like Parkinson's disease, Huntington's disease, Alzheimer's disease and myotonic dystrophy.[25-28] ncRNAs are classified as small ncRNAs or long ncRNAs, according to their length. ncRNAs include microRNAs (miRNAs) from 19 to 24 bp, small nucleolar RNAs (snoRNAs) from 60 to 300 bp, PIWI interacting RNAs (piRNAs) from 26 to 31 bp, transcription initiation RNAs (tiRNAs) from 17 to 18 bp, promoter associated small RNAs (PASRs) from 22 to 200 bp, circular RNAs (circRNAs) with variable size and TSS associated RNAs (TSSa-RNAs) from 20 to 90 bp. Long noncoding RNAs (lncRNAs) are longer than 200 nucleotides and are a very heterogeneous group of molecules. They may be classified according to their genome localization and/or by their orientation (sense, antisense, bidirectional, intronic or intergenic lncRNAs).[29-31] In the following section, we address the most studied ncRNAs in relation to myotonic dystrophy.

miRNAs IN MYOTONIC DYSTROPHY

MicroRNAs (miRNAs) are endogenous small (21–23 nt in length) non-coding RNAs that control gene expression at the posttranscriptional level. They down-regulate gene expression by imperfect pairing with complementary sites within transcript sequences and suppress their translation, stimulate deadenylation and degradation, or induce target cleavage.

Given that DM1 has its principal symptoms at the level of skeletal muscle, studies tend to focus on muscle tissue. As a result, several changes in muscle-specific miRNAs (myo-miRNAs) have been reported for DM1. Myo-miRNAs regulate muscle function and adaptation during development (proliferation, differentiation, quiescence, regeneration) and disease.[20,32,33] In a recent study of biopsies of DM1 patients, a reduced expression of miRNA-1, miRNA-133a, and miRNA-133b was observed in the patients' muscle. Previous studies have proposed that miRNA-1 is a member of the group of «degenerative miRNAs» which may be mediators of cell death, contributing to apoptotic/necrotic myofiber loss. And they also found an overexpression of the considered «regenerative miRNA», miRNA-206, in DM1 muscle, as previously reported in DM1 and DMD.[14,23,34]

In a recent study, muscle-specific miRNAs were explored, which could be considered objective and circulating biomarkers of the efficacy of rehabilitation in DM1. Rehabilitation was used to counteract muscle atrophy and improve muscle function. In the study they have shown a significant downregulation of myo-miRNAs and myostatin after physical rehabilitation in parallel with the improvement of clinical functional tests. A significant downregulation of miR-1, miR-133a, miR-133b, and miR-206 after 12 weeks of endurance training and a decrement of miR-133a after strength training were observed. The results suggest that miR-1, miR-133a, miR-133b, miR-206, and myostatin

might be considered circulating objective biomarkers of rehabilitation efficacy in DM1, supporting the clinical outcome measures.[35]

In another study, it was shown that miR-1 and miR-335 were up-regulated whereas miR-29b, miR-29c and miR-33 were down-regulated in DM1 biopsies. Moreover, they found that potential miR-1 targets are significantly up-regulated due to a miR-1 subcellular localization which was severely disrupted, altering its function. miR-1 is a crucial regulator not only of myogenic differentiation, but also of muscle cell excitability. It is suggested that miR-1 plays an important role in DM1.[20]

An important molecule involved in myogenesis is Twist-1. Congenital DM1 cells which have a defective differentiation program have low levels of MyoD and miR-206 but high Twist-1 levels. Twist-1 is an important molecule involved in myogenesis, which belongs to the family of bHLH transcription factors. However, in mouse C2C12 myoblasts and in human embryonic stem cell (HESC)-derived embryoid bodies, Twist-1 is found to inhibit muscle cell differentiation. miR-206 is a negative regulator of Twist-1 and promotes muscle cell differentiation. Therefore, the MyoD -miR-206-Twist-1 pathway is compromised in DM1 cells that exhibit a defective differentiation program.[36,37]

In DM1, cardiac muscle is also affected, and several miRNA families are deregulated in patient heart tissues. It has been found that CUG exp RNA expression leads to an up-regulation of miR-21 and down-regulation of miR-29, miR-30 and miR-133 family members, and this study shows that tight reciprocal relationship between gain and loss of these miRNAs that target genes have a critical role in the core network in DM1 cardiac fibrosis. The miR-23a/b family regulates post-transcriptional loss of CELF1 protein during mouse postnatal heart development; reduced levels of miR-23a and miR-23b in DM1 heart tissue are expected to result in an overall increase in CELF1 protein levels, thus contributing to mis-regulation of CELF1 splicing targets. A select set of miRNAs in DM1, including miR-1, is down-regulated due to a reduced MEF2 transcriptional program. Mef2c is a transcriptional factor essential for direct reprogramming of cardiac fibroblasts into induced cardiomyocytes, and the loss of Mef2 activity causes deregulation of many miRNAs and mRNAs in a DM1 cardiac cell (culture model) and heart tissue (mouse model).[12,38]

At the molecular level, one of the best-characterized trans-dominant effects induced by the CUGexp-RNAs in DM1 is the mis-regulation of alternative splicing of a subset of premRNAs. More recently, Charlet's team has described a novel function of the RNA binding protein MBNL1 as a regulator of the micro-RNA miR-1 biogenesis. A Predictive bioinformatic analysis indicates that pre-miR-1 have potential MBNL1 binding site. Based on this observation, a miRNome analysis of human muscle cells showed a significant alteration of miR-1 expression in DM1 cells. MBNL1 binds to a UGC motif located within the loop of pre-miR-1 and competes for the binding of LIN28, which promotes pre-miR-1 uridylation by TUT4 and blocks dicer processing.[14,21] Consequently, miR-1 loss in the heart causes increased expression of connexin 43 and CACNA1C, as they are targets of miR-1. CACNA1C and connexin 43 encode the main calcium-and gap-junction channels in heart, respectively, and their mis-regulation could contribute to cardiac dysfunction, such as conduction defect observed in the DM1 patients.

Recently, microRNAs have been found to be present at significant levels in extracellular body fluids, including blood serum and plasma. Perfetti et al. identified a signature of nine deregulated miRNAs in plasma samples of DM1 patients and suggested that these miRNAs can be used as diagnostic biomarkers for DM1, and the muscle-specific miR-133a was included in these miRNAs.[19] In another study the muscle-specific miRNAs miR-1, miR-133a, miR-133b and miR-206 were detected in the sera isolated from DM1 patients and their levels were found to be significantly higher in progressive DM1 patients compared to non-progressive DM1 patients; this implies that these muscle-specific miRNAs presumably leak from the degraded muscle tissue during muscle wasting and enter the blood circulation of the patient. However, the increase in the serum levels of myo-miRNAs observed in DM1 patients was not correlated with disease severity.[23]

Prior to these studies, deregulated plasma miRNAs in DM1 were validated. They confirmed that 8 miRNAs out of 12 were significantly deregulated in DM1 patients, including non-muscle specific miRNAs, namely miR-140, -27b, -454 and 574; indeed, since DM1 is a multisystemic disorder, it is possible that the tissue of origin of these miRNAs might not be the skeletal muscle.[39] However, a more recent study in serum, shows that only miR-21 had a significantly different expression between controls and patients. This study took previously reported miRNAs into account and there were discrepancies in the results, which can be attributed to the statistical methods or

differences in the experimental procedures used in each study.[40] Therefore, it is necessary to carry out more studies regarding the deregulation of miRNAs in patient serum due to their potential importance as biomarkers.

Regarding therapeutic studies involving miRNAs in DM1, a recent study performed in a Drosophila model focused on silencing specific miRNAs and regulating the expression of muscleblind and demonstrated that the silencing of miR-277 or miR-304 in muscle using sponge constructs achieved muscleblind upregulation, which was sufficient to rescue characteristic DM1 model phenotypes such as missplicing events, reduced lifespan, and muscle atrophy.[41] A summary of deregulated miRNAs in DM1 is showed in *Table 1*.

A study of transcriptome in an inducible glial cell model for DM1, the MIO-M1 $CTG_{(648)}$ cells, revealed for the first time a dysregulated levels of miRNAs and lncRNAs in central nervous system.[42] Except for miR-222 in muscle,[43] the deregulated miRNAs found in this study had not been previously reported in DM1. An analysis revealed an involvement of the altered miRNAs in processes with relevance to CNS function, specifically in nervous system development.

The deregulated expression of miR-4288, miR-222, miR103, miR-298 and miR-448 found in the DM1 model is shared with other neurodegenerative conditions such as Alzheimer's disease (AD) and Huntington's disease (HD).[44-47] CELF3 was identified as a miR-298 target, while CELF5 and CELF6 were both predicted as miR-448 targets. MBNL1 was revealed as one of the predicted miR-4288 targets. Considering the central role playing by MBNL and CELF proteins in the DM1 pathogenic mechanism, further studies are required to explore the functional consequences of the indicated dysregulated miRNAs. The ontological analysis also revealed a regulation of the immune/inflammatory response mediated by miRNAs in MIO-M1 $CTG_{(648)}$ cells.[42]

LncRNAs IN DM1

The deregulation of lncRNAs in DM1 has not yet been studied. Recent studies have demonstrated the importance of lncRNAs in various pathologies, including neuromuscular diseases.[25] However, a recent study classifies an antisense transcript from the DM1 locus as an lncRNA. A previous study performed by Tapscott and coworkers was the first to report that there is an antisense transcription emanating from the adjacent SIX5 regulatory region that extends into the insulator element and is converted into 21 nucleotides. The authors suggest that it is involved in local modifications of chromatin.[5]

However, a recent study by Gudde et al. shows that transcripts of this antisense (DM1-AS) occur as very low-abundance RNAs of different lengths.[18] This antisense transcript contains alternative polyadenylation sites, and alternative splicing may remove the (CAG)n repeat from the longer DM1-AS RNAs. The results of this study indicate that DM1-AS RNAs are produced extending downstream from the insulator element formed by the CTCF-binding sites. Bioinformatics analysis and RT-qPCR approaches have shown that DM1-AS transcripts are produced in essentially all cell types and tissues. Despite a mild increase in DM1-AS expression in patients, the findings indicate that DM1-AS transcripts occur roughly 5–50-fold less frequently than DMPK mRNA molecules, with variation in this ratio dependent on cell or tissue type.

The extremely low expression of these transcripts has important implications for the function of DM1-AS RNA and for its potential contribution to DM1 pathology. Presence of expanded DM1-AS RNA in the nucleus and in the cytoplasm would allow involvement in the formation of toxic nuclear RNP aggregates and in the generation of RAN translation products in the cytoplasm. RNP foci containing expanded (CAG) n RNA have indeed been reported for DM1 cells. Homopolymeric RAN peptides, which could be formed from DM1-AS RNA with expanded (CAG)n tracts, may exert proteotoxicity at a very low concentration, like formation of abnormally aggregated protein complexes around prion-protein cores in only some cells in a tissue population. This study also proposes that DM1-AS RNAs could engage in formation of dsRNA molecules by hybridization to complementary sequences in *DMPK* transcripts. Such an event might trigger toxic dsRNA-responsive kinase signaling with possible immune effects or abnormal effects of aberrant repeat-containing siRNA, formed after DICER processing of the dsRNA.

Another theory is that DM1-AS transcripts may play a structural role in local chromatin organization in the DM1 locus in the nucleus. Given the evidence from their previously mentioned work, Gudde et al. conclude that primary and processed DM1-AS transcripts belong to the heterogeneous class of lncRNAs, because they share many signatures with this type of RNA. lncRNAs, like mRNAs, may be subject to posttranscriptional

processing, including capping, polyadenylation and splicing. Despite their naming, it has now become clear that at least some lncRNAs still do encompass an ORF and can undergo translation.[18,48-51]

In an inducible glial cell model for DM1, the MIO-M1 $CTG_{(648)}$ cells, previously mentioned they found dysregulated levels of lncRNAs. However, the role these ncRNAs play in the pathogenesis of DM1 is still unknown.[42]

siRNAs IN DM1

siRNA is derived from long double-stranded RNA molecules (including RNAs arising from virus replication, transposon activity or gene transcription), which can be cut by the DICER enzyme into RNA fragments of 19-24 nt, with the resulting RNA fragments exercising their functions when loaded onto Argonaute (AGO) proteins *(Figure 1)*. Recent studies showed that siRNA can lead to transcriptional gene silencing in cells by means of DNA methylation and histone modification in cells.[16,27,52-54]

The previously mentioned study by Cho, et al. found that the antisense transcription of the DM1 locus can be converted into 21 nucleotide fragments (siRNAs) that recruit histone methyltransferases, HP1, and DNA methyltransferases, with associated conversion of the region to heterochromatin. In a

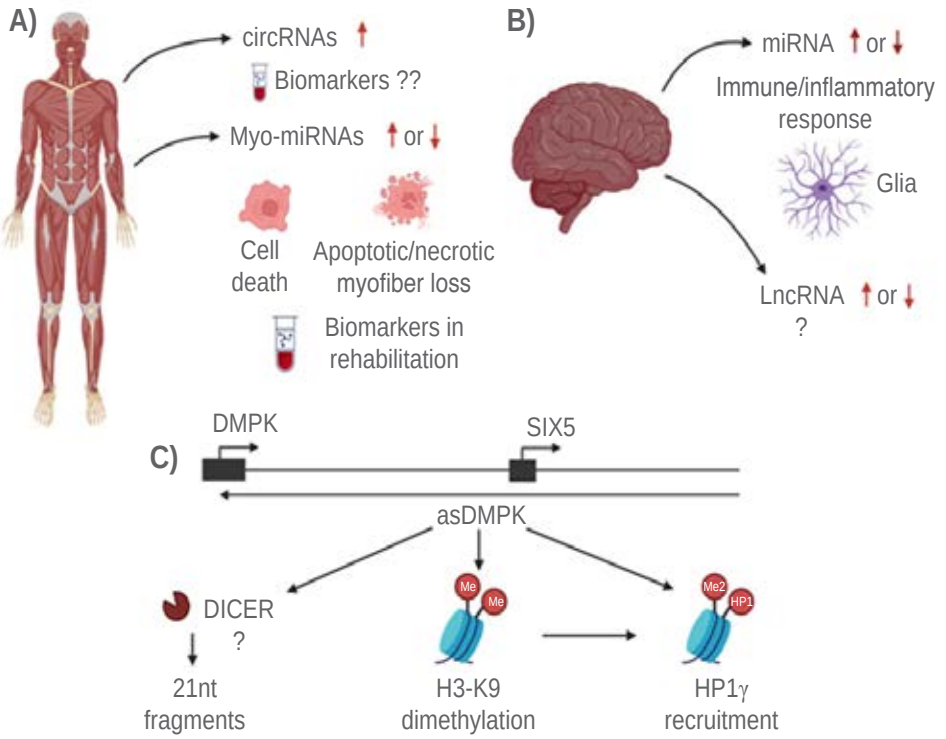

Figure 1: A) Interestingly, upregulated circRNAs have been reported in muscle from DM1 patients, however, require more comprehensive analyses in order to determine whether circRNAs are reliable biomarkers and could be used for prognosis and as therapeutic agents and targets in DM1. The dysregulation of muscle-specific miRNAs (Myo-miRNAs) in DM1, opens the possibility of using these ncRNAs as biomarkers of rehabilitation, or indicators of cell death, apoptotic/necrotic or myofiber loss. **B)** ncRNAs play a very important role at the CNS level. In a glia DM1 model, the dysregulation of miRNAs that may be involved in the immune/inflammatory response has been described. Dysregulation in the levels of lncRNAs was also reported, although the role of these lncRNAs in glia has not been described. **C)** Model described by Cho, et al. where in the WT allele is associated with bidirectional transcription (forming an antisense RNA, asDMPK), the formation of 21nt RNA fragments (possibly mediated by DICER), H3-K9 dimethylation and recruitment of HP1g in the region of CTG repeats. This mechanism suggests local modifications of chromatin, mediated by siRNAs.

Table 1: miRNAs and circRNAs deregulated in DM1.

Sample	Reporter changes	Method	Comments	References
Human muscle biopsies from the vastus lateralis	Up: miR-206	RT-qPCR	Seven unrelated patients, aged 30-50 years	Gambardella, et al. (2010)[61]
Human muscle biopsies from the vastus lateralis	Up: miR-206 Down: miR-1, miR-133a, miR-133b	RT-qPCR	Twelve unrelated patients, aged 19-52 years	Fritegotto, et al. (2017)[22]
Human muscle biopsies from biceps	Up: miR-1, miR-335 Down: miR-29b, miR-29c, miR-33	RT-qPCR	Fifteen unrelated patients, aged 38 ± 17 years	Perbellini, et al. (2011)[20]
Serum	Up: miR-1, miR-133a, miR-133b, miR-206	RT-qPCR	Twenty-three patients	Koutsoulidou, et al. (2015)[23]
Plasma (validation)	Up: miR-1, miR-133a, miR-133b, miR-206, miR-140-3p, miR-574, miR-454 Down: miR-27b	RT-qPCR	One hundred three patients, aged 44.1 ± 1.3 years	Perfetti, et al. (2016)[39]
Serum (validation)	None (validation)	RT-qPCR	Twenty-six patients, aged 26-61 years	Fernández-Costa, et al. (2016)[40]
Drosophila i(CTG)480 transgenic line Human skeletal muscle biopsies (biceps, vastus and deltoid)	Up: one miRNA Down: nineteen miRNAs Down: miR-1, miR-7, miR-10a	SOLiD™ 3 sequencing	Deregulation of miR-1, miR-7 (given their conservation in humans) Five patients, aged 47 ± 5 years	Fernández-Costa, et al. (2013)[13]
Mouse model (EpA960; MCM) Human heart tissues	Postnatal down: miR-23a, miR-23b Deregulated: 54 miRNAs Down: twenty miRNAs	RT-qPCR	Eight patients, aged 26-55 years	Kalsotra, et al. (2014)[12]
Human heart left ventricles samples	Down: miR-1	RT-qPCR	Eight adults	Rau, et al. (2011)[14]
Human muscle biopsies from the biceps brachii	Up: miR-208a, miR-381 Down: miR-193b-3p	Gene chip human exon 1.0 ST array (Affymetrix)	miRNAs validated in DM2 were also tested in an age-and sex-matched cohort of DM1 patients	Greco, et al. (2012)[62]
Myoblast cell lines, muscle biopsy and samples from the *HSA*^{LR} transgenic mouse model	Increase of circRNAs level	Next generation sequencing	Human cell lines three DM1 and controls. Human muscle biopsies five DM1 and six controls. Mouse muscles ten DM1 and ten controls	Czubak, et al. (2019)[59]
Muscle tissue biopsies, myogenic cell lines	Increased circular fraction: CDYL, HPK3, RTN4_03 and ZNF609	RNA seq	Muscle tissue biopsies from biceps brachii of 30 DM1 and 29 sex- and age-matched control individuals	Voellenkle, et al. (2019)[60]
Inducible glial cell model (MIO-M1-CTG(648) cells)	111 deregulated genes; 9.1% of ncRNAS	Clariom D Arrays for human samples	Four experimental groups, 3 biological replicates	Azotla-Vilchis CN, et al. (2021)[42]

more recent study that aimed to define the potential effects of bi-directional transcription, expanded CAG repeat transcripts were co-expressed with the DM1 CTG repeats. This resulted in dramatically enhanced toxicity concomitant with the generation of triplet repeat-derived siRNAs. Both CAG and CUG strands can be processed into ~21 nt small RNAs when co-expressed and small RNAs derived from both strands are methylated in a *Hen1*-dependent manner. These results suggest that both CAG and CUG small RNAs can be loaded into mature, holo-RISCs presumably due to the symmetrical thermodynamic properties of the repeat small RNA duplex. This study confirms that two CAG containing genes, *atx2* and *tbp* are targets of the triplet repeat-derived siRNAs. These results suggest that bi-directional transcription of the repeat region in diseases like DM1 may confer additional components of pathogenicity due to deleterious interactions between the two-overlapping repeat-containing transcripts through the generation and activity of triplet repeat-derived siRNAs. These effects may include downregulating the expression of other genes containing CAG repeats. This suggests that both expanded CAG and CTG are required for triplet repeat-derived siRNA generation and toxicity *in vivo*.[16,55]

circRNAs IN DM1

circRNAs are single stranded circularized molecules which are mainly generated from the precursor mRNA backsplicing process.[56] Recently, strict tissue, cell, developmental, and age expression specificity has been demonstrated for several circRNAs, supporting the hypothesis that these transcripts are of functional importance. The biology and function of most circRNAs are still poorly recognized, but it is becoming increasingly clear that specific circular RNAs function as sponges for miRNAs and proteins, affecting RNA splicing and regulating transcription.[57] A recent study tested the hypothesis of circRNAs downregulation in DM1, known to be a burden with functional deficiency of MBNL proteins and dysregulation of alternative splicing.[58] Czubak et al., selected 20 validated circRNAs and analyzed their expression levels in several experimental systems, including human myoblast cultures and skeletal muscle biopsy samples from patients and healthy individuals. In addition, they used muscles from the HAS transgenic mouse model of DM1. However, they found no downregulation of the analyzed circRNAs in DM1 samples compared with those in non-DM1 samples. Therefore, these results question the role of MBNL proteins in circRNA biogenesis in muscles. Interestingly they discovered a consistent increase in circRNA levels.[59] The obtained data in this study do not confirm the hypothesis regarding the link between MBNL sequestration and disrupted circRNA biogenesis in DM1, but do not exclude the possibility of the existence of individual circRNAs that are regulated by MBNLs. An increased level of circRNAs in DM1 skeletal muscle has also most recently been reported in another study by Voellenkle et al *(Table 1)*.[58-60]

The most recent studies, which identified upregulation of circRNAs in DM1 patients' skeletal muscles, require more comprehensive analyses in order to determine whether circRNAs are reliable biomarkers and could be used for prognosis and as therapeutic agents and targets in DM1.[58] However, the role of individual circRNAs altered in DM1 and their global function in DM1 pathogenesis remain to be determined.

CONCLUSIONS

ncRNAs play important roles in healthy and disease tissues. It has been found that ncRNAs, particularly miRNAs, participate in the development, differentiation, and regeneration of muscle tissue in DM1. The potential role of some miRNAs as DM1 biomarkers has been revealed from serum patient's studies. More recent studies have illuminated the more detailed role of the initially described antisense DM1 RNA, now classified as a lncRNA, with a potential role in the formation of siRNAs, chromatin modifying and RAN translation mechanisms.

Nonetheless, lncRNA have not been described in DM1, and it would therefore be interesting to investigate the role they play in this disease.

It is important to mention the importance that ncRNAs can have as therapeutic targets, as it has been observed that their modulation can reverse some phenotypic traits of the disease, and an understanding of the mechanisms that involve ncRNAs can provide more candidates for genic therapies.

It appears that ncRNAs play an important role in DM1, adding new elements to the previously described mechanisms, that improve our understanding of this complex disease, leaving much to still be discovered.

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