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Myotonic Dystrophy Type 1: Focus on the RNA Pathology and Therapy

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1. Introduction

Almost 100 years ago, Steinert (1909), Batten and Gibb (1909), independently described Myotonic Dystrophy type 1 (DM1) that is now recognized as the most common form of muscular dystrophies in adults and the second most common type of muscular dystrophy after Duchenne Muscular Dystrophy, affecting 1 in 8000 individuals globally (Harper, 1989). DM1 is a genetic disorder, which is inherited in an autosomal dominant fashion (the mutation in one copy of the affected allele is enough to cause the disease). Although the disease affects mainly the skeletal muscle, it is considered a multi-systemic disorder with variable clinical symptoms affecting skeletal muscle, heart, and the central nervous system (CNS) (Larkin & Fardaei, 2001). Individual patients with DM1 are often identified as having congenital, juvenile or adult-onset disease based on the age of symptom onset. Congenital cases display the most severe phenotype and face a neonatal mortality rate of 25% (Harper, 1989).

The involvement of skeletal muscle in DM1 is highly characteristic and largely unwavering. Skeletal muscle in DM1 displays progressive weakness and wasting, myotonia and pain. Moreover, at the early stages of the disease, DM1 patients exhibit facial and neck flexion muscle weaknesses. Also, ptosis and weakness of eye and mouth closure are classical facial changes observed in DM1 patients. Weakness of neck flexion is an early sign, and patients may notice difficulty in lifting their heads from the pillow or experience tendency for the head to fall backwards during acceleration of the vehicle in which they are traveling (Machuca-Tzili et al., 2005). At a later stage in the course of the disease, distal weakness in the limbs, affecting particularly the finger flexors causes substantial disability. Less marked but often occurring, the weakness of ankle dorsiflexion causes foot drop. The combination of facial muscle weakness (with ptosis) and distal muscle weakness in DM1, even in the absence of myotonia, does not occur in any other disease (Ranum & Day, 2002). Respiratory failure due to the weakness of the respiratory muscles can also occur. This may be lethal in some cases, and is presented mainly in DM1 patients that experienced anesthesia or suffered from various chest infections (Machuca-Tzili et al., 2005). Myotonia is demonstrable in most

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symptomatic adults, whatever their symptoms. The commonest symptom of myotonia is difficulty in relaxing the grip. Myotonia can also affect the facial muscles, tongue, and other bulbar muscles, causing problems when talking, chewing and swallowing. Muscle pain is clearly independent to myotonia and more common in the lower limbs, where myotonia is usually not observed (Machuca-Tzili et al., 2005; Ranum & Day, 2002). Biopsies of DM1 muscle show a markedly increased variation in fibre diameter that ranges from 10μm to greater than 100μm. Severely atrophic fibres have pygnotic nuclei with minimal remaining contractile elements. DM1 muscle observations also showed ring fibre and central nuclear chains. Moreover, ATPase staining of the affected muscle sections showed atrophy of type 1 fibres. Finally, basophilic regenerating fibres, splitting fibres, fibrosis and adipose deposition are common muscle abnormalities of DM1, always depending on the extent of muscle involvement (Vihola et al., 2003).

Antrioventricular and intraventricular conduction abnormalities are very common in DM1 and require regular monitoring. DM1 patients were shown to be more vulnerable to cardiac conduction abnormalities than impaired myocardial function. Atrial fibrillation, ventricular arrhythmias and cardiomyopathy are also very common abnormalities in DM1 affected individuals (Phillips & Harper, 1997). Sudden death, due to heart block, is not common in DM1, but does occur in severe DM1 patients, as a result of extreme sinus bradycardia or tachyarrhythmia, necessitating the use of pacemakers and implantable defibrillators by these patients (Colleran et al., 1997). There is extensive evidence for CNS involvement in DM1. Cognitive impairment / mental retardation, specific patterns of psychological dysfunction and personality traits, are widely recognized features of congenital and juvenile DM1 affected patients. In addition to the above, DM1 patients develop CNS white matter and cerebral blood flow abnormalities (Ogata et al., 1998). Central hypersomnia, another recognized CNS effect of DM1, appears to occur in adulthood. Excessive daytime sleepiness is very common and in some cases very disabling. Apathy, epilepsy, stroke and parkinsonism are rarely observed in adult DM1 patients, but are fairly common in congenitally affected patients (Rubinsztein et al., 1997). Posterior capsular, iridescent, multicoloured opacities are regularly seen in DM1 patients. Cataracts can be detected in DM1 patients at a very early stage in the course of the disease by slit-lamp examination. When vision is significantly impaired, surgical intervention is required (Klesert et al., 2000). Irritable bowel-like symptoms such as constipation, diarrhea, colicky abdominal pain and pseudo-obstruction are extremely common in DM1. The upper gastrointestinal tract is affected in later stages of the disease and can cause dysphagia and aspiration resulting in serious chest infections that can cause morbidity and mortality of DM1 patients. DM1 patients display a large variety of endocrine abnormalities. Testicular failure, hypotestosteronism and oligospermia are associated with the reduced fertility of these patients. Laboratory observations have also shown a reduction in the serum levels of IgG and IgM and as a result, hypogammaglobulinemia can affect these patients. Insulin resistance is also seen in DM1 patients, but even in severe cases, type 2 diabetes clinical symptoms are not linked with the disease, whilst recent evidence supports that there is alteration in the normal functioning of the insulin receptor (Moxley et al., 1984).

DM1 is caused by an unstable expansion of CTG repeats in the 3′-untranslated region (3′ UTR) of the *dystrophia myotonica protein kinase (DMPK)* gene on chromosome 19q13.3 (Aslanidis et al., 1992; Brook et al., 1992; Davies et al., 1983). The number of CTG repeats is

in the range of 5–35 in the normal population and increases to between 50 and several thousand in DM1 patients. The variation in the number of CTG repeats is closely related to the phenomenon of 'anticipation': The number of CTG repeats can increase through successive generations, due to mitotic and meiotic instabilities (Martorell et al., 1999; Monckton et al., 1995). Interesting is also the fact that DM1 patients might show difference in the number of CTG repeats from one cell to another (genetic heterogeneity). 3'UTR is a region that is transcribed into RNA but not translated into protein therefore the CTG expansion at the DM1 locus does not alter the protein sequence encoded by DMPK. The mechanism of pathogenesis in DM1 is different from other genetic disorders. Three different mechanisms of pathogenesis have been proposed to explain how repeat expansions in the *DMPK* gene result in DM1 (Tapscott & Thornton, 2001):

i. The mutant RNA accumulates in discrete foci in the cell nucleus rather than being transported to the cytoplasm (Davis et al., 1997; Taneja et al., 1995), where translation of mRNA into protein normally takes place. According to experimental evidence this causes deficiency in the production of DMPK (DMPK Haploinsufficiency) (Jansen et al., 1996; Reddy et al., 1996).
ii. The second mechanism of pathogenesis, involves the disruption of the expression and function of DMPK neighboring genes. Scientific evidence showed that the expanded CTG repeats interfere with the nucleosome assembly and therefore on the total chromatin structure. This process disrupts and prevents the binding of necessary factors for the expression of neighboring genes (Klesert et al., 1997; Wang & Griffith, 1995; Westerlaken et al., 2003).
iii. Lastly, evidence suggests that the molecular pathogenesis of DM1 is to a great extent due to the downstream effects of the retention of the mutant DMPK transcripts in the nucleus. Some of these include the inhibition of myogenesis and the defective splicing of cellular RNA molecules (Mankodi et al., 2000; Miller et al., 2000; Timchenko et al., 1996).

This book chapter will focus mainly on the pathology which arises from nuclear RNA retention and the therapeutic approaches against it. The correlation between the CTG repeat size and the severity of the disease, suggests that the repeats represent the major cause of DM1 pathogenesis. After investigations of the mutant DMPK transcripts, it was found that these form nuclear foci that are enclosed within the nucleus of the affected cells (Amack & Mahadevan, 2001; Taneja et al., 1995). Moreover, it was postulated that these nuclear foci might contribute to DM1 pathogenesis, perhaps by disrupting the transport of mRNA from DMPK and/or other genes to the cytoplasm (Alwazzan et al., 1999; Klesert et al., 1997; Otten & Tapscott, 1995; Taneja et al., 1995). Further on, cell models expressing the CTG repeat expansion, showed also nuclear localization of mutant RNA foci and extended myogenic defects (Amack & Mahadevan, 2004). These nuclear RNA foci were also shown to interact with nuclear RNA binding proteins and subsequently alter the regulation or localization (Fardaei et al., 2002; Philips et al., 1998). Mutant DMPK RNA foci were found to interact with the CUG-binding protein (Timchenko et al., 1996) and three different forms of the muscleblind binding protein (MBNL) (Ho et al., 2005). Scientific evidence, using knockout MBNL or CUGBP animal models revealed similarities to the DM1 pathophysiology, including cardiac, endocrine system and muscle abnormalities (Kanadia et al., 2003; Kanadia et al., 2003; Roberts et al., 1997).

2. RNA pathogenesis

2.1 RNA nuclear retention

Mutant DMPK alleles are transcribed in the nucleus to produce RNA molecules containing expanded CUG repeats. The expanded CUG transcript folds back on itself to form stable duplex hairpin structures. Napierala and Kryzosiak provided evidence that hairpins indeed exist in the DMPK RNA fragments containing 11–49 CUG repeats and that the stability of these structures increases with the repeat length (Napierala & Krzyzosiak, 1997). Koch and Leffert, generated secondary structures of partial and full-length DMPK mRNAs carrying variable numbers of CUG triplet repeats (up to 500) (Koch & Leffert, 1998). They suggested that CUG hairpins are the most stable structures formed and also that the DMPK mRNAs are sterically impeded from transport through nuclear pores, by giant hairpins or hairpin clusters formed by CUG repeats above a limit size (44 or less) (Koch & Leffert, 1998). Further thermal melting and nuclease mapping studies indicated that CUG repeats form highly stable hairpins (Tian et al., 2000). Michalowski et al., used electron microscopy to provide the first visual evidence that the DMPK mRNA expansion forms an RNA hairpin structure (Michalowski et al., 1999). Visualization of large RNAs containing up to 130 CUG repeats revealed perfect double-stranded RNA segments whose lengths were that expected for duplex RNA (Michalowski et al., 1999). The duplex segments were highly stable, since the hairpin structures reformed rapidly during the electron microscopy mounting procedures when the RNAs were boiled and quickly cooled, even under low salt conditions (Michalowski et al., 1999).

RNA harboring CUG repeat expansion impose dominant-negative effects by aberrantly interacting and recruiting RNA-binding proteins thus leading to nuclear retention of the mutant transcript and the formation of ribonuclear inclusions known as RNA foci. Taneja et al., used fluorochrome-conjugated and digoxigenin-conjugated oligonucleotide probes to analyze the intracellular localization of DMPK transcripts in fibroblasts derived from DM patients and normal individuals and showed a striking difference in the nuclear distribution of the DMPK transcripts, a difference that was verified in a muscle biopsy from an affected individual (Taneja et al., 1995). Davis et al., generated "myoblasts" by *MyoD* retroviral infection of DM1 fibroblast lines and showed that mutant *DMPK* transcripts were abundant in myoblasts, but could not contribute to kinase production, as the transcripts were quantitatively retained within myoblast nuclei (Davis et al., 1997). Terminally differentiated myoblasts contained no cytoplasmic mutant transcripts; instead, they formed stable, long-lived clusters that were tightly linked to the nuclear matrix (Davis et al., 1997). Myoblasts and myotubes isolated from patients with congenital myotonic dystrophy (CDM1) also showed abnormal retention of mutant RNA in nuclear foci (Furling et al., 2001).

2.2 CUG binding protein 1 (CUGBP1)

Proteins of the CELF family are a group of proteins extensively studied for their implication in DM1 pathogenesis. CELF (CUGBP and ETR-3-like factors) proteins are a family of structurally related RNA-binding proteins involved in various aspects of RNA processing including alternative splicing, translation and mRNA stability. The first member of the CELF family, CELF1/CUGBP1, was identified in investigating the molecular mechanisms of DM1 (Gallo & Spickett, 2010). Timchenko et al. reported that CUG binding protein 1

(CUGBP1) binds specifically to CUG triplet repeated sequences (Timchenko et al., 1996; Timchenko et al., 1996). Electron microscopy studies showed that CUGBP1 is primarily a single-stranded RNA-binding protein that has a binding preference for CUG-rich RNA elements but not double-stranded CUG hairpins (Michalowski et al., 1999). Moreover, CUGBP1 was visualized to localize to the base of the RNA hairpin and not along the stem (Michalowski et al., 1999). In a yeast three-hybrid system CUGBP1 was found to associate with long CUG trinucleotide repeats ($(CUG)_{(11)}(CUG)_{(12)}$), but not with short repeats ($(CUG)_{(12)}$) (Takahashi et al., 2000). However, using a combination of indirect immunofluorescence to detect endogenous proteins and overexpression of proteins with green fluorescent protein (GFP) tags it has been shown that CUGBP1 does not co-localise with triplet repeat foci in DM1 fibroblast cell lines (Fardaei et al., 2001).

Experiments in tissue culture and analysis of DM1 patients demonstrated that RNA CUG repeats directly affect expression and activity of CUGBP1 in DM1 myoblasts, heart, and skeletal muscle tissues (Timchenko et al., 2001). Specifically, the formation of CUGBP1 · CUG RNA complexes is accompanied by increased CUGBP1 protein stability and subsequent elevation of CUGBP1 (Timchenko et al., 2001). Furthermore, nuclear CUGBP1 levels have been found increased in DM1 patients, compared to normal subjects (Timchenko et al., 2001). These observations suggest that abnormal activation of CUGBP1 is related to DM1 pathogenesis. Furthermore, in a transgenic mice model, overexpression of CUGBP1 in heart and skeletal muscle, produced DM1-like symptoms such as central nuclei, chains of nuclei, centralized nicotinamide adenine dinucleotide (NADH) reactivity and mis-splicing (Ho et al., 2005). De Haro et al., generated a *Drosophila* model of DM1 that showed degenerative phenotypes in muscle and eye tissue and key histopathological features of the DM1, including accumulation of the expanded transcripts in nuclear foci (de Haro et al., 2006). Using this model they showed that by increasing the levels of CUGBP1 degeneration is deteriorated even though CUGBP1 distribution is not altered by the expression of the expanded triplet repeat (de Haro et al., 2006). Wang et al., generated an inducible and heart-specific DM1 mouse model expressing expanded CUG RNA in the context of DMPK 3' UTR that recapitulated pathological and molecular features of DM1 including dilated cardiomyopathy, arrhythmias, systolic and diastolic dysfunction, and mis-regulated alternative splicing (Wang et al., 2007). Combined *in situ* hybridization and immunofluorescent staining for CUGBP1 protein expressed in heart, showed increased protein levels specifically in nuclei containing foci of CUG repeat RNA (Wang et al., 2007).

Although the molecular mechanisms for increased CUGBP1 is not completely understood, Kuyumcu-Martinez et al. reported that the expression of mutant DMPK-CUG-repeat RNA results in hyperphosphorylation and stabilization of CUGBP1 through the inappropriate activation of the protein kinase C (PKC) pathway, in DM1 tissues, cells, and a DM1 mouse model (Kuyumcu-Martinez et al., 2007). Experiments performed in C2C12 mouse cell line showed that expression of a mutant DMPK 3'-UTR containing 960 CUG repeats is sufficient to increase expression and stability of an mRNA encoding the potent proinflammatory cytokine, tumor necrosis factor (TNF), which was found elevated in DM1 patients serum (Mammarella et al., 2002; Zhang et al., 2008). Moreover, activation of the protein kinase C (PKC) pathway also stabilized the TNF transcript. These results suggest that the elevated serum TNF seen in DM1 patients may be derived from muscle where it is induced by expression of toxic DMPK RNA (Zhang et al., 2008). In a more recent study, Koshelev et al.

used tetracycline-inducible CUGBP1 and heart-specific reverse tetracycline trans-activator transgenes in order to express human CUGBP1 in adult mouse heart (Koshelev et al., 2010). Up-regulation of CUGBP1 was sufficient to reproduce molecular, histopathological and functional changes observed in DM1 patients and in a DM1 mouse model thus supporting a role for CUGBP1 up-regulation in DM1 pathogenesis (Koshelev et al., 2010). In another mouse model, Ward et al. overexpressed CUGBP1 and showed that mice reproduced molecular and physiological defects of DM1 tissue, suggesting that CUGBP1 has a major role in DM1 skeletal muscle pathogenesis (Ward et al., 2010).

2.3 Muscleblind (MBNL) family proteins

Muscleblind (MBNL) family proteins, initially identified by Miller et al. were selectively associated with CUG repeat expansions and named as triplet repeat expansion (EXP) double-stranded (ds) RNA-binding proteins (Miller et al., 2000). Human EXP proteins are found to be orthologous to the *Drosophila* MBNL proteins, which are required for terminal differentiation of photoreceptors and muscle cells (Artero et al., 1998; Begemann et al., 1997). The alternative splicing factor MBNL1 binds to pyrimidine-rich pre-mRNAs containing YGCY motifs and promotes either the inclusion or the exclusion of alternative exons depending on the 5′ or 3′ localization of *cis*-regulatory elements (Goers et al., 2010). All three isoforms of MBNL family proteins (MBNL/MBNL1, MBLL/MBNL2 and MBXL/MBNL3) co-localize with the nuclear mutant RNA foci in DM1 cells, presumably diverting them from their normal cellular functions (Wojciechowska & Krzyzosiak, 2011). The biological significance of the interaction between mutant RNA and MBNL-family proteins has been manifested through the disruption of alternative splicing which is a characteristic feature of DM1 pathogenesis (Wojciechowska & Krzyzosiak, 2011). Fardaei et al. investigated for the first time the localization of MBNL (EXP) protein with mutant DMPK transcripts (Fardaei et al., 2001). Using indirect immunofluorescence to detect endogenous proteins and overexpression of GFP-tagged MBNL, they showed that MBNL forms foci in DM1 fibroblast cell lines and co-localises with the foci of expanded repeat transcripts in the nuclei of DM1 cells (Fardaei et al., 2001). The binding of MBNL1 with expanded CUG repeats was further verified in DM1 muscle biopsy tissues (Mankodi et al., 2003) and in a yeast three-hybrid system (Kino et al., 2004). Overexpression of MBNL1 *in vivo* using a recombinant adeno-associated viral vector rescued disease-associated muscle myotonia and aberrant splicing of specific gene transcripts, characteristic of DM1 skeletal muscle supporting the hypothesis that loss of MBNL1 activity is a primary pathogenic event in the development of the disease (Kanadia et al., 2006).

Analysis of the expression pattern of the mouse *Mbnl1*, *Mbnl2*, *Mbnl3* and *Dmpk* genes during embryonic development revealed a striking overlap between the expression of *Dmpk* and the *Mbnl* genes during development of the limbs, nervous system and various muscles, including the diaphragm and tongue (Kanadia et al., 2003). In 2003, Kanadia et al. generated the first MBNL knockout mouse model for DM1 (Kanadia et al., 2003). The disruption of the mouse *Mbnl1* gene led to muscle, eye and RNA splicing abnormalities which are characteristics of DM1 disease (Kanadia et al., 2003). Examination of DM1 post-mortem brain tissue by FISH indicated that the mutant DMPK mRNA is widely expressed in cortical and subcortical neurons and accumulated in discrete foci within neuronal nuclei (Jiang et al., 2004). Moreover, MBNL family proteins were recruited into the RNA foci and a subset of

neuronal pre-mRNAs showed abnormal regulation of alternative splicing suggesting that central nervous system impairment in DM1 may result from a deleterious gain-of-function by mutant DMPK mRNA (Jiang et al., 2004). In 2005, Mankodi et al. provided evidence that accumulation of expanded CUG repeats in nuclear foci was associated with sequestration of MBNL proteins and abnormal regulation of alternative splicing in cardiac muscle tissue from DM1 patients (Mankodi et al., 2005).

In a *Drosophila* model of DM1 expressing CTG repeats in the 3′-UTR of a marker gene CUG repeats form discrete ribonuclear foci in muscle cells that co-localize with MBNL (Houseley et al., 2005). Moreover, MBNL was also revealed as having a previously unrecognized role in stabilizing CUG transcripts (Houseley et al., 2005). In another *Drosophila* model of DM1 that shows degenerative phenotypes in muscle and eye tissue as well as key histopathological features of the DM1, including accumulation of the expanded transcripts in nuclear foci and their co-localization with MBNL1 protein, reduced levels of MBNL1 aggravate the muscle and eye phenotypes of DM1 flies whereas MBNL1 overexpression suppresses the degenerative phenotypes (de Haro et al., 2006). *Mbnl2*-deficient mice developed myotonia, skeletal muscle pathology consistent with human DM1 and defective CLCN1 mRNA splicing in skeletal muscle, supporting the hypothesis that MBNL proteins and specifically MBNL2 contribute to the pathogenesis of human DM1 (Hao et al., 2008). These results are consistent with the notion that *Mbnl1* deficiency alone is not sufficient to fully replicate the human DM1 phenotype (Kanadia et al., 2003). An additional mouse DM1 model with inducible and skeletal muscle-specific expression of 960 CTG repeats in the context of DMPK exon 15 recapitulated many findings associated with DM1 skeletal muscle, such as nuclear foci with MBNL1 protein co-localization, mis-splicing, myotonia, characteristic histological abnormalities, and increased CUGBP1 protein levels (Orengo et al., 2008). Importantly, this DM1 mouse model exhibited severe muscle wasting, which has not been reported previously in models in which MBNL1 depletion was the main feature (Orengo et al., 2008). More recently, Machuca-Tzili et al. generated an *mbnl2* knockdown zebrafish model, which exhibits features of DM (Machuca-Tzili et al., 2011). They showed that loss of zebrafish mbnl2 function causes muscle defects and splicing abnormalities of clcn1 and tnnt2 transcripts, similar to those observed in DM1 patients (Machuca-Tzili et al., 2011).

Wheeler et al., showed that CUG expanded RNA is also expressed in subsynaptic nuclei of muscle fibers and in motor neurons in DM1 patients, causing sequestration of MBNL1 protein in both locations (Wheeler et al., 2007). Additionally, in a transgenic mouse model, expression of CUG expanded RNA at high levels in extrajunctional nuclei replicates many features of DM1, including myotonia, spliceopathy, internal nuclei, ring fibers, and sarcoplasmic masses, but the toxic RNA is poorly expressed in subsynaptic nuclei and mice fail to develop denervation-like features of DM1 myopathology (Wheeler et al., 2007). These findings suggest that subsynaptic nuclei and motor neurons are at risk for DM1-induced mis-splicing, which may affect function or stability of the neuromuscular junction (Wheeler et al., 2007). MBNL1 protein was also found in the human brain, and consists of several isoforms, as shown by RT-PCR and sequencing. In the brain tissue of DM1 patients, a fetal isoform of MBNL1 was found overexpressed (Dhaenens et al., 2008). The expression of this fetal isoform can also be reproduced by the ectopic expression of long CUG repeats *in vitro* (Dhaenens et al., 2008).

2.4 Mis-regulation of alternative splicing

At the molecular level, one of the best-characterized trans-dominant effects induced by the mutant *DMPK* RNAs in DM1 is the mis-regulation of alternative splicing of a subset of pre-mRNAs. Alternative splicing is a process by which the exons of the transcribed pre-mRNA are reconnected in multiple ways to give rise to different mRNAs which are in turn translated into different protein isoforms. To date, more than twenty transcripts have been found to be mis-spliced in different tissues of DM1 patients (Klein et al., 2011).

2.4.1 CUGBP1 and MBNL1 significance in alternative splicing mis-regulation

Mis-splicing events observed in DM1 result from an inappropriate regulation of alternative splicing due to altered activities of splicing regulators such as CUGBP1 and MBNL1. The biological significance of CUGBP1 overexpression and MBNL family proteins sequestration to nuclear RNA foci has been manifested through the disruption of alternative splicing, which is a characteristic feature of DM1 pathogenesis. Muscle wasting and weakness, heart problems and insulin resistance are associated with aberrant alternative splicing of a range of pre-mRNAs.

2.4.1.1 Cardiac troponin T (cTNT)

The first mis-regulation of alternative splicing described in DM1 was the abnormal inclusion of exon 5 in cardiac troponin T (cTNT) in cardiac muscle (Philips et al., 1998). CUGBP1 was found to bind to the human cTNT pre–mRNA and regulate its alternative splicing. Splicing of cTNT was disrupted in DM1 striated muscle and in normal cells expressing CUG expanded RNAs (Philips et al., 1998). Transgenic mice with a targeted deletion of *Mbnl1* exon 3 (E3) (*Mbnl1*$^{\Delta E3/\Delta E3}$) in adult heart showed abnormal retention of the *cTNT* "fetal" exon 5, as was observed in DM1 (Kanadia et al., 2003; Philips et al., 1998). Ho et al., involved for the first time all three MBNL family members with mis-regulation of alternative splicing in DM1 (Ho et al., 2004). MBNL proteins were found to act antagonistically to CELF proteins on the human and chicken cTNT pre-mRNAs (Ho et al., 2004). MBNL1 binds a common motif near the human and chicken cTNT alternative exons within intronic regions, which appear to be single stranded (Ho et al., 2004). Furthermore, CELF and MBNL proteins bind to distinct *cis*-elements and minigenes containing CELF- or MBNL-binding site mutations thus showing that regulation by one family does not require responsiveness to the other (Ho et al., 2004). However, modified cTNT minigenes made nonresponsive to the *trans*-dominant effects of CUG repeat RNA still respond to MBNL depletion, suggesting that CUG repeat RNA affects splicing by a mechanism more complex than MBNL depletion alone (Ho et al., 2004).

2.4.1.2 Insulin receptor (IR)

Three years after the discovery of the first pre-mRNA that is mis-spliced in DM1, Savkur et al., described the second pre-mRNA that undergoes mis-regulation of alternative splicing in DM1 skeletal tissue (Savkur et al., 2001). Alternative splicing of the insulin receptor (IR) pre-mRNA was aberrantly regulated in DM1, resulting in predominant expression of the lower-signaling non-muscle isoform (IR-A) of the receptor (Savkur et al., 2001). IR-A also predominates in DM1 skeletal muscle cultures, which exhibit a reduced responsiveness to the metabolic effects of insulin (Savkur et al., 2001). Furthermore, the aberrant regulation of

IR alternative splicing was reproduced in normal cells by the expression of CUG-repeat RNA (Savkur et al., 2001). Additionally, overexpression of CUGBP1 also induced a switch to IR-A in normal cells (Savkur et al., 2001). The CUGBP1 protein mediates this switch through an intronic element located upstream of the alternatively spliced exon 11, and specifically binds within this element *in vitro* (Savkur et al., 2001). The research group suggested a model in which increased expression CUGBP1 splicing regulator contributes to insulin resistance observed in DM1 by affecting IR alternative splicing (Savkur et al., 2001).

In addition to CUGBP1, IR alternative splicing was found to be regulated by MBNL1 (Ho et al., 2004). Down-regulation of MBNL1 and MBNL2 in normal myoblasts resulted in abnormal splice pattern observed in DM1 (Dansithong et al., 2005). Moreover, CUGBP1 was found to regulate the equilibrium of splice site selection by antagonizing the facilitatory activity of MBNL1 and MBNL2 on IR exon 11 splicing in a dose-dependent manner (Dansithong et al., 2005). Rescued experiments in DM1 myoblasts demonstrated that loss of MBNL1 function is the critical event, whereas CUGBP1 overexpression plays a secondary role in the aberrant alternative splicing of IR RNA in DM1 (Dansithong et al., 2005). Therefore, these experiments demonstrated that MBNL1 sequestration is the primary determinant of the IR pre-mRNA abnormal splicing in DM1 myoblasts (Dansithong et al., 2005).

2.4.1.3 Chloride channel - 1 (ClC-1)

In a transgenic mouse model of DM1, the expression of expanded CUG repeats reduced the transmembrane chloride conductance to an extent sufficient to account for myotonia (Mankodi et al., 2002). These mice showed abnormal splicing of pre-mRNA encoding chloride channel – 1 (ClC-1), the main chloride channel in skeletal muscle, resulting in loss of ClC-1 protein from the surface membrane (Mankodi et al., 2002). Furthermore, the induction of abnormal ClC-1 splicing, the corresponding loss of ClC-1 protein from the muscle membrane, and the development of myotonia were tightly correlated with the level of expanded CUG repeats in different transgenic lines (Mankodi et al., 2002). Additional to the mice models, similar effects on CLC-1 splicing and protein accumulation in muscle tissue from patients with DM1 (Mankodi et al., 2002). These findings suggest that mis-regulation of ClC-1 pre-mRNA alternative splicing is an important factor that leads to myotonia observed in DM1 patients. In an additional study, loss of ClC-1 mRNA and protein due to aberrant splicing of the ClC-1 pre-mRNA was detected in DM1 skeletal muscle tissue (Charlet et al., 2002). Specifically, the majority of ClC-1 mRNAs contained premature termination codons due to retention of intron 2 or inclusion of two novel exons between exons 6 and 7 (Charlet et al., 2002). CUGBP1, which is found elevated in DM1 striated muscle, bound to the ClC-1 pre-mRNA, and overexpression of CUGBP1 in normal cells reproduced the aberrant pattern of ClC-1 splicing observed in DM1 skeletal muscle (Charlet et al., 2002). In particular, CUGBP1 induced retention of intron 2 by binding to a U/G-rich motif common to other pre-mRNA targets of CUGBP1 thus suggesting that increased CUGBP1 activity in DM1 causes aberrant regulation of ClC-1 alternative splicing (Charlet et al., 2002).

2.4.1.4 Tau

Tau protein belongs to the family of microtubule-associated proteins whose transcript undergoes complex regulated splicing in the mammalian nervous system. They are

essentially expressed in neurons where their essential function is to regulate the microtubule network. In the adult human central nervous system, alternative splicing of exons 2, 3 and 10 of the single *tau* gene transcript gives six tau isoforms (Sergeant et al., 2001). The tau isoforms aggregated in DM1 brain lesions consists mainly of the shortest human tau isoform suggesting that the $(CTG)_n$ expansion is altering the processing of tau pre-mRNA splicing and gives rise to symptoms such as dementia (Sergeant et al., 2001).

2.4.1.5 Myotubularin-related 1 (MTMR1)

The *myotubularin-related 1 (MTMR1)* gene belongs to a highly conserved family of phosphatases with at least 11 isoforms in humans (Buj-Bello et al., 2002). One of the transcripts resulting from MTMR1 alternative slicing is muscle-specific and is induced during myogenesis both *in vitro* and *in vivo*, and represents the major isoform in adult skeletal muscle (Buj-Bello et al., 2002). *MTMR1* splicing pattern was found strikingly altered in cultured muscle cells, in skeletal muscle from patients with congenital myotonic dystrophy (CDM1) and in DM1 muscle biopsies (Buj-Bello et al., 2002; Santoro et al., 2010).

2.4.1.6 Ryanodine receptor 1 (RyR1) and sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) 1 or 2

Ryanodine receptor 1 (RyR1) and sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) 1 or 2 which are the main sarcoplasmic reticulum regulators of intracellular Ca^{2+} homeostasis in skeletal muscle cells (Kimura et al., 2005). The fetal variants, ASI(-) of RyR1 which lacks residue 3481-3485, and SERCA1b which differs at the C-terminal were found significantly increased in skeletal muscles from DM1 patients and a transgenic mouse model of DM1 thus suggesting that aberrant splicing of RyR1 and SERCA1 mRNAs might contribute to impaired Ca^{2+} homeostasis in DM1 muscle (Kimura et al., 2005).

2.4.1.7 Myocyte enhancer factor 2 (Mef2)

MBNL3 regulates the splicing pattern of the muscle transcription factor myocyte enhancer factor 2 (Mef2) (Lee et al., 2010). MBNL3 antagonizes muscle differentiation by disrupting the expression of (+)β isoform of Mef2D which is transcriptionally more active (Lee et al., 2010). Using a DM1 cell culture model and DM1 patient tissue, they provided evidence that expression of CUG expanded RNAs can lead to an increase in MBNL3 expression accompanied by a decrease in Mef2D β-exon splicing (Lee et al., 2010). These studies suggest that an increase in MBNL3 activity may play a role in the skeletal muscle degeneration experienced by DM1 patients (Lee et al., 2010).

2.4.1.8 Bridging integrator-1 (BIN1)

Bridging integrator-1 (BIN1) is a myc box-dependent-interacting protein involved in tubular invaginations of membranes and is required for the biogenesis of muscle T tubules, which are specialized skeletal muscle membrane structures essential for excitation-contraction coupling (Fugier et al., 2011). Mutations in the *BIN1* gene cause centronuclear myopathy, which shares some histopathological features with myotonic dystrophy (Jungbluth et al., 2008). BIN1's function is regulated by alternative splicing which was found altered in skeletal muscle samples of people with CDM1 and DM1 (Fugier et al., 2011). Particularly, MBNL1 was detected to bind the BIN1 pre-mRNA and regulate the alternative splicing of the exon 11 (Fugier et al., 2011). Sequestration of MBNL1 by expanded CUG repeats in DM1 patients resulted in expression of an inactive form of BIN1 lacking phosphatidylinositol 5-

phosphate-binding and membrane-tubulating activities (Fugier et al., 2011). Consistent with a defect of BIN1, muscle T tubules found altered in DM1 patients, and membrane structures were restored upon expression of the normal splicing form of BIN1 in muscle cells of such individuals (Fugier et al., 2011). Finally, reproducing BIN1 splicing alteration in mice was sufficient to promote T tubule alterations and muscle weakness, a predominant feature of DM1 (Fugier et al., 2011).

2.4.1.9 Myomesin 1 (MYOM1)

Myomesin 1 (MYOM1) is a constituent of the M band of the sacromere which is the basic unit of a muscle (Lange et al., 2005). The fact that MYOM1 is a structural constituent of muscle suggests that it could be involved in muscle impairment in patients with DM1. Koebis et al. used exon array and identified aberrant inclusion of *MYOM1* exon 17a as a novel splicing abnormality in DM1 muscle (Koebis et al., 2011). A cellular splicing assay using a *MYOM1* minigene revealed that MBNL and CELF family proteins function as *trans*-acting factors in the alternative splicing of *MYOM1* exon 17a (Koebis et al., 2011). Expression of expanded CUG repeat impeded MBNL1 activity but did not affect CUGBP1 activity on the splicing of *MYOM1* minigene (Koebis et al., 2011). These results suggested that the downregulation of MBNL proteins should lead to the abnormal splicing of *MYOM1* exon 17a in DM1 muscle (Koebis et al., 2011).

2.5 Myogenic defects

DM1-associated molecular events, such as abberant recruitment of RNA-binding proteins into ribonuclear foci and mis-splicing, could eventually provoke alterations in global cell function. Notably, a critical disorder of DM1 that distinguishes it from other muscular dystrophies is the defects observed in muscle differentiation. Fetal muscle development is affected in fetuses with congenital myotonic dystrophy whereas muscle regeneration is compromised in adult patients (Amack & Mahadevan, 2004).

Initial experiments performed in the mouse myoblast cell line C2C12 showed that overexpression of the mouse *dmpk* gene leads to markedly inhibition of both fusion and terminal differentiation of the cell line (Okoli et al., 1998). Further experiments performed on C2C12 cell line showed that overexpression of human DMPK mRNA caused a marked inhibition of terminal differentiation accompanied by a reduction of myogenin mRNA levels (Sabourin et al., 1997). These results suggest that overexpression of the DMPK 3'-UTR may interfere with the expression of muscle-specific mRNAs leading to a delay in muscle terminal differentiation (Sabourin et al., 1997). Amack et al. used reporter assays to provide unambiguous evidence that the expression of the mutant *DMPK* 3′-UTR mRNA with $(CUG)_{200}$ selectively inhibited myogenic differentiation of C2C12 myoblasts (Amack et al., 1999). In agreement, overexpression of DMPK 3'-UTR including either wild-type or expanded CTG repeats resulted in aberrant and delayed muscle development in fetal transgenic mice and displayed muscle atrophy at 3 months of age. Moreover, primary myoblast cultures from both wild-type and expanded CTG repeat mice showed reduced fusion potential with greater reduction observed in the expanded repeat cultures (Storbeck et al., 2004). Interestingly, the differentiation defect was confirmed in muscle cell cultures derived from DM1 fetuses and patients (Furling et al., 2001; Timchenko et al., 2001).

Molecular studies have begun to uncover the effect of the mutant DMPK mRNA on myogenesis inhibition. Various studies implicate CUGBP1 protein with myogenic impairment in DM1, since it is a key regulator of translation of proteins that are involved in muscle development and differentiation. Timchenko et al. reported that cultured myoblasts isolated from DM1 patients failed to permanently withdraw from the cell cycle when stimulated to differentiate (Timchenko et al., 2001). Skeletal muscle cells from DM1 patients failed to induce cytoplasmic levels of CUGBP1, while normal differentiated cells accumulate CUGBP1 in the cytoplasm (Timchenko et al., 2001). In normal cells, CUGBP1 up-regulates p21 translation during differentiation by binding to a GC-rich sequence located within the 5' region of p21 mRNA (Timchenko et al., 2001). DM1 cultured cells failed to accumulate CUGBP1 in the cytoplasm thus leading to a significant reduction of p21 and to alterations of other proteins responsible for the cell cycle withdrawal (Timchenko et al., 2001). In normal cells, activity of cdk4 declines during differentiation, whereas in DM1 cells cdk4 is highly active during all stages of differentiation (Timchenko et al., 2001). Furthermore, DM1 cells do not form Rb/E2F repressor complexes that are abundant in differentiated cells from normal individuals (Timchenko et al., 2001). These data provide evidence for an impaired cell cycle withdrawal in DM1 muscle cells and suggest that alterations in the CUGBP1 activity causes disruption of p21-dependent control of cell cycle arrest (Timchenko et al., 2001). Another study showed that CUGBP1 is phosphorylated by different kinases during myoblast proliferation and differentiation and that phosphorylation of CUGBP1 at different sites directs CUGBP1 to different mRNA targets (Salisbury et al., 2008). Specifically, Akt kinase and cyclinD3-cdk4/6 phosphorylate CUGBP1 during proliferation and differentiation, respectively (Salisbury et al., 2008). Cyclin D3-cdk4-mediated phosphorylation of CUGBP1 increases the interactions of CUGBP1 with eIF2 during normal myogenesis, a pathway found to be reduced in DM1 cells (Salisbury et al., 2008). Moreover, ectopic expression of cyclin D3 in DM1 cells enhances fusion of DM1 myoblasts and leads to the correction of differentiation (Salisbury et al., 2008). A more recent study, showed that human skeletal muscle satellite cells isolated from fetal congenital DM1 patients bearing large CTG expansions (>3000) secrete prostaglandin E2 (PGE(2)) that inhibits the fusion of normal myoblasts in culture by decreasing the intracellular levels of calcium (Beaulieu et al., 2011). Authors suggest that the delay in muscle maturation observed in congenital DM1 patients may result, at least in part, from an altered autocrine mechanism (Beaulieu et al., 2011).

3. Therapeutic approaches for DM1

3.1 Restoring MBNL1 and CUGBP1 protein activity

It is widely accepted that MBNL and CUGBP play a major role in the pathogenesis of DM1 and are therefore targets for the reversal of the defective splicing and subsequent alleviation of symptoms in the disease. MBNL activity is compromised due to its sequestration in RNA foci. Therefore an approach to increase its expression levels and hence its activity could serve as a potential route for restoring alternative splicing. Kanadia et al. used intramuscular injection with an AAV (adeno-associated virus) expressing MBNL1 protein in a transgenic mouse model of DM1. This transgenic mouse model carries the human skeletal *a-actin (HSA)* gene modified by insertion of 250 CTG repeats within the 3′ untranslated region. These mice develop severe myotonia and dystrophic muscle features characteristic of DM1 disease. Overexpression of MBNL1 saturated the expanded CUG binding sites of the mutant DMPK

transcripts and free MBNL1 was able to cause the reversal of muscle hyperexcitability, myotonia and spliceopathy (Kanadia et al., 2006). These results, demonstrated that the elevated expression of MBNL1 alone was sufficient to rescue myotonia, a key pathological feature of DM1, and aberrant splicing of specific gene transcripts, characteristic of the DM1 skeletal muscle. In a parallel study, de Haro et al. used a *Drosophila* model of DM1 expressing mRNA transcripts containing 480 CUG repeats, which accumulate in nuclear foci and show degenerative phenotypes in muscle (muscle wasting) and eye tissue (disorganization and fusion of the ommatidia as well as loss and duplication of inter-ommatidial bristles) as well as other key histopathological features of DM1. Furthermore, this DM1 model shows altered levels of MBNL1 and CUGBP1, as observed in DM1 pathogenesis. Overexpression of MBNl1 in this DM1 model showed to suppress the muscle and eye phenotypes of DM1. Interestingly, expanded RNA transcripts that accumulated in nuclear foci within muscle cells were decreased in flies expressing the mutant RNA transcripts that also overexpress MBNL1 (de Haro et al., 2006).

CUGBP1 has been shown to be up-regulated in DM1 as a result of PKC activation and subsequent CUGBP1 protein hyperphosphorylation and stabilization (Kuyumcu-Martinez et al., 2007). Wang et al. created a heart specific Tamoxifen-inducible mouse model containing 960 CTG repeats within the last exon of the DMPK. These mice exhibited high mortality, conduction abnormalities, and systolic and diastolic dysfunction as well as molecular changes seen in DM1 patients, such as increased levels of CUGBP1, colocalization of MBNL1 with RNA foci and reversion of splicing to embryonic patterns. Blocking of PKC activity, using a specific PKC inhibitor (Ro-31-8220), in this heart-specific DM1 mouse model ameliorated several DM1 symptoms, including cardiac conduction defects and contraction abnormalities (Wang et al., 2009). The inhibitor also reduced the splicing defects regulated by CUGBP1, but not those regulated by MBNL1, suggesting distinct roles for these proteins in DM1 cardiac pathogenesis (Wang et al., 2009). As previously described, DM1 mouse models showed elevated levels of CUGBP1 that leads to a delay of muscle development and differentiation. Salisbury et al. presented evidence that two signal transduction pathways regulate CUGBP1 activity in normal muscle and that these pathways are altered in DM1 cells. CUGBP1 was found to be phosphorylated by different kinases, during myoblast proliferation and differentiation and that phosphorylation of CUGBP1 at different sites directs CUGBP1 to different mRNA targets. Moreover, cyclin D3-cdk4-mediated phosphorylation of CUGBP1 increases the interactions of CUGBP1 with eIF2 during normal myogenesis. Furthermore, it was found that cyclin D3-cdk4 pathway is reduced in DM1 cells and that the normalization of cyclin D3 expression in DM1 cells leads to the correction of differentiation (Salisbury et al., 2008).

Overexpression of MBNL1 and downregulation of CUGBP1 or modulation of their counterparts showed encouraging results towards the development of rational therapies for DM1. These two key proteins play a major role in the pathogenesis of the disease and any attempt to normalize their function will be beneficial.

3.2 Targeting the mutant DMPK transcripts

A promising gene therapy approach is to target the mutant DMPK transcripts. Most of the attempts to eliminate toxic *DMPK* transcripts in DM1 cells and animal models used catalytic

RNA (ribozymes) (Langlois et al., 2003; Phylactou et al., 1998), chemically modified antisense oligonucleotides (Furling et al., 2003; Mulders et al., 2009) and siRNA duplexes (Langlois et al., 2005).

Ribozymes are RNA molecules that adopt a tertiary structure and function as catalysts. The hammerhead, hairpin and hepatitis delta virus (HDV) ribozyme motifs can be characterized by their ability for self-cleavage of a particular phosphodiester bond. Hammerhead ribozymes have the ability to suppress gene expression through specific cleavage of RNA molecules (Phylactou et al., 1998; Tedeschi et al., 2009). Langlois et al. designed a hammerhead ribozyme with significant accessibility to a specific target site within the 3′ UTR of the DMPK mRNA. Utilizing this system, a significant reduction of mutant and normal DMPK 3′ UTR transcripts was observed. Furthermore, these human DM1 myoblasts showed a significant reduction of nuclear RNA foci and a partial restoration of insulin receptor isoform B expression. This study demonstrated for the first time intracellular ribozyme-mediated cleavage of nuclear-retained mutant DMPK mRNAs, providing a potential gene therapy agent for the treatment of myotonic dystrophy (Langlois et al., 2003). Beside degradation of their targets, ribozymes can lead to splicing events that replace target RNA with embedded sequences. Group I Intron ribozymes can be characterized by their capacity for self-splicing by cleavage and ligation of phosphodiester bonds (Cech, 1990; Fiskaa & Birgisdottir, 2010). Group I intron ribozymes can be designed to act in *trans* by recognition and separation of RNA molecules in a sequence specific manner, and ligation of a new RNA sequence to the separated RNA molecules. In their 1998 study, Phylactou et al. created a group I intron ribozyme to cleave, *in vitro*, a DMPK RNA containing 12 repeats and replace them with 5 repeats. Furthermore, it was shown that similar splicing was able to be achieved in human cultured fibroblasts (Phylactou et al., 1998). Another promising approach is to target the mutant DMPK transcripts with antisense oligonucleotides. In 2003, Furling et al. showed that by infecting DM1 cells in culture with an adenoviral vector expressing an antisense RNA to the CUG repeat sequence, the mutant DMPK mRNA was significantly reduced. In addition, effective restoration of human DM1 myoblast functions such as myoblast fusion and the uptake of glucose was achieved (Furling et al., 2003). Furthermore, DM1 cells expressing the antisense RNA indicated a correction of CUGBP1 expression in infected DM1 cells. Muscle differentiation and insulin resistance in DM1 were found to be in close proximity with the misregulation of CUGBP1 protein levels.

Alternative approaches towards targeting the mutant DMPK transcripts in DM1, include the use of chemically modified antisense oligonucleotides. A recent report showed convincingly the therapeutic effect of 2-*O*-methyl phosphorothioate modified $(CAG)_7$ oligonucleotides in DM1 mouse models and in patient myoblast cultures (Mulders et al., 2009). The addition of 2-O-methyl groups to a phosphorothioate-modified oligonucleotide confirms increased stability of binding and reduced nonspecific effects. Local administration of the modified oligonucleotide in skeletal muscle resulted in approximately 50% reduction of expanded *DMPK* RNA. As a result, RNA foci were also reduced and defective splicing corrected. Such findings demonstrate that a low $(CUG)_n$ RNA dosage can still be beneficial to patients and be an attractive therapeutic approach. Myotonia is one of the key features of DM1 and is associated with abnormal alternative splicing of the muscle-specific chloride channel (ClC-1) and reduced conductance of chloride ions in the sarcolemma. Wheeler et al. developed a morpholino antisense oligonucleotide targeting the 3′ splice site of ClC-1 exon 7a and reversed the defect of ClC-1 alternative splicing in two mouse models of

DM1. The levels of ClC-1 mRNA and eventually protein were found to be upregulated. Moreover, treated mice had a fully functional chloride channel and lack myotonia (Wheeler et al., 2007).

In one approach to inhibit the sequestration of MBNL1 with the expanded CUG repeats, Wheeler et al. used a $(CAG)_{25}$ antisense oligonucleotide morpholino. Antisense morpholinos are unable to cause the cleavage of their target RNAs. *In vitro*, these morpholinos were able to bind to the expanded CUG repeats forming a stable RNA-morpholino heteroduplex that was able to block the formation of MBNL1-RNA complexes and disrupt complexes that had already formed. *In vivo*, intramuscular injection and electroporation of the $(CAG)_{25}$ antisense oligonucleotide morpholino in a transgenic mouse model that accumulate expanded CUG RNA and MBNL1 protein in nuclear foci in skeletal muscle, caused the reduction of nuclear foci and redistribution of MBNL1 protein. 14 weeks after treatment, myotonia was significantly reduced and ClC-1 function restored. The same approach was used to test the effect of this morpholino in an mbnl-1 deficient mouse, which mimics most of the splicing abnormalities of DM1. The morpholino had no effect, confirming that the morpholino specifically acts on the expanded repeats, which are not present in the mbnl1 knockout model (Wheeler et al., 2009).

RNAi has also been used successfully to degrade mutant *DMPK* transcripts. SiRNA duplexes induce the specific cleavage of target RNAs in mammalian cells. Although most of the RNAi applications rely on the cytoplasmic effect of these molecules, it has been shown that RNAi phenomena can occur in the nucleus of primary DM1 cells by targeting nuclear retained *DMPK* mRNAs (Langlois et al., 2005). Krol et al. attempted to target particularly long hairpin structures formed from the interactions of CUG repeats rather than the targeting of nascent RNA in general. In this study it was demonstrated that these long CUG repeat hairpins are under the control of a ribonuclease Dicer, involved in the RNA interference pathway whose main function is to induce the fragmentation of double-stranded RNA duplexes into shorter duplexes, which then act as endogenous siRNAs and trigger the downstream silencing effect. Furthermore, it was shown that the transduction of synthetic $(CAG)_7$ siRNAs into DM1 patient fibroblasts, leads towards a selective reduction of mutant transcripts containing long CUG repeats (Krol et al., 2007).

An alternative approach to confront the toxicity of nuclear RNA retention is to block the binding of RNA-binding proteins to mutant *DMPK* transcripts using small chemical molecules with high affinity towards the mutant *DMPK* transcripts. The binding of these molecules on the mutant *DMPK* transcripts should prevent the binding and therefore the sequestration of RNA-binding proteins, such as MBNL1, and restore aberrant splicing (Mastroyiannopoulos et al., 2010). Several approaches have been performed with the most promising of these being the use of pentamidine, a small molecule that binds to the expanded CUG RNA sequence with high affinity and specificity causing reduction of CUG repeat foci formation and relieves MBNL1 sequestration (Warf et al., 2009). Moreover, pentamidine reversed the mis-splicing of 4 different pre-mRNAs affected in DM1 (Warf et al., 2009). Gareiss et al. used resin-bound dynamic combinatorial chemistry in order to identify the compounds that are able to inhibit MBNL1 binding to expanded CUG RNA. Screening of 11,325 members yielded several molecules with significant selectivity for binding to CUG repeat RNA. These compounds were also able to inhibit the interaction of expanded CUG with MBNL1 *in vitro* (Gareiss et al., 2008). In another report, the design of

high affinity ligands that bind to expanded CUG and CAG repeats and inhibit the formation of RNA-protein complexes that are implicated in DM1 was described (Pushechnikov et al., 2009). Similarly, Garcia-Lopez et al. identified molecules that aim to target toxic CUG RNA transcripts when applied on a *Drosophila* model of DM1. By performing a positional scanning combinatorial peptide library screen, a D-amino acid hexapeptide (ABP1) that reduced CUG-induced toxicity in fly eyes and muscles was identified. Furthermore, ABP1 reversed muscle histopathology and splicing misregulation of MBNL1 targets in DM1 model mice. *In vitro*, ABP1 was found bound to CUG hairpins and induced a switch to a single-stranded conformation (Garcia-Lopez et al., 2011). In another study, Arambula et al. created a ligand with high nanomolar affinity to CUG RNA or CTG DNA repeats. This ligand is a triaminotriazine-acridine conjugate designed to hydrogen bond to both U's or T's in the U-U or T-T mismatch, interactions observed between binding of multi CUG and CTG repeats. This ligand was found to destabilize the interactions of MBNL1 with multi CUG repeats (Arambula et al., 2009).

3.3 Induction of mutant DMPK transcripts nuclear export

Few studies have attempted to determine whether the export of mutant DMPK transcripts is beneficial for the disease. The export of mutant DMPK RNA transcripts from the nucleus to the cytoplasm will have as a result the recovery of these cells from DM1 pathogenic events. The export of mRNA from the nucleus is a highly ordered and complicated procedure that implicates several molecules. As previously described, mutant DMPK RNA carrying long CUG repeats form haipin structures which then interact with proteins such as MBNL1 and CUGBP1. These interactions most possibly prohibit the export of these transcripts to the cytoplasm and cause nuclear retention in the form of foci.

Mastroyiannopoulos et al. have demonstrated nuclear export of mutant DMPK 3'-UTR transcripts by introducing a viral post-transcriptional regulatory element. The WPRE (Woodchuck post-transcriptional regulatory element) has been widely used as an enhancer during transgene expression (Lee et al., 2005). It is also known to enhance gene expression through stimulation of nuclear RNA export (Donello et al., 1998). WPRE was inserted downstream of the 3'-end of a mutant DMPK 3'-UTR sequence and was shown to bypass nuclear entrapment. With the use of fluorescence *in situ* hybridization it was shown that the mutant DMPK transcripts that carried the WPRE sequence were localized mainly in the cytoplasm of C2C12 cells in the form of foci. WPRE mediated nuclear export enhanced muscle cell differentiation (Mastroyiannopoulos et al., 2005) and more specifically initial fusion of myoblasts (Mastroyiannopoulos et al., 2008). In another study, cardiac cells, identified from a transgenic mouse in which 400 CTG repeats were positioned downstream of the reporter *LacZ* gene and upstream of the bovine growth hormone polyadenylation signal, localized CUG aggregates exclusively in the cytoplasm of cells (Dansithong et al., 2005). Aggregation of CUG RNAs within the cytoplasm resulted both in MBNL1 sequestration and in approximately 2-fold increase in both nuclear and cytoplasmic CUGBP1 levels. Significantly, and despite these changes, RNA splice defects were not observed, and functional analysis revealed only subtle cardiac dysfunction. These results demonstrate that the presence of mutant DMPK transcripts in the cytoplasm in the form of foci is insufficient to elicit DM1 defects. Interestingly, Garcia-Lopez et al. described a transgenic *Drosophila* model expressing expanded CTG repeats which exhibit an extended

DM1 phenotype. Such as, muscle degeneration, ribonuclear formation, interactions with muscleblinds including misregulated alternative splicing of muscle genes and CUG depended central nervous system alterations. Genetic screens and functional assays on this *Drosophila* model identified the RNA export factor Aly as one of the causes of the phenotype. It has been shown that the Aly phenotype has a close relationship to mRNA export factors and EJC (exon junction complex) components. Mutations in Aly were shown to be associated with nuclear accumulation of CUG repeats. It is therefore important to study further the mRNA export pathway implicated in DM1 and identify candidate targets for repairing nuclear retention of the DMPK transcripts (Garcia-Lopez et al., 2008). Finally, a report proved in a very convincing way the benefit of exporting mutant DMPK transcripts to the cytoplasm. Wheeler et al. attempted to reverse the myotonic dystrophy symptoms in a well-studied animal model by interfering with the MBNL1 and CUG RNA hairpins interaction. The authors used a 25-nt antisense molecule, composed of CAG repeats, in order to prevent the interaction between MBNL1 and expanded CUG repeats by forming a heteroduplex with the hairpins. The oligonucleotide caused elimination of foci and released the trapped transcripts to the cytoplasm. Moreover, the antisense oligonucleotide repaired the defect of mis-splicing and restored the CLCN1 function and myotonia. This paper provided proof-of concept about the therapeutic potential of molecules that prevent the deleterious interactions between proteins and RNA in diseases.

4. Discussion / conclusions

In conclusion, almost 20 years after the discovery of DM1 mutation there is a very good understanding of the pathogenic mechanisms involved in DM1. As previously stated, the mutant DM1 RNA transcripts generate most of the pathological aspects of DM1. The retention of DMPK transcripts as ribonuclear inclusions in the nucleus of DM1 cells is considered to be an important pathogenic mechanism of the disease (Mankodi et al., 2000). A growing body of evidence showed that pleiotropic effects of aberrant interactions between mutant DMPK 3' UTR transcripts and RNA-binding proteins alter the metabolism of 'target' messenger RNAs (Ho et al., 2005; Jiang et al., 2004; Timchenko et al., 1996; Timchenko et al., 2001; Timchenko et al., 2004). Members of the muscleblind family (MBNL, MBXL and MBLL), which usually regulate mRNA splicing, have been shown to colocalize with the ribonuclear inclusions. The nuclear interactions of the MBNL proteins with the mutant DMPK 3' UTR transcripts, have shown to affect the splicing of various mRNAs, such as the insulin receptor (IR), troponin T (cTNT) and the muscle-specific chloride channel (ClC-1). Another RNA-binding protein, implicated in DM1 mechanism of pathogenesis, is CUGBP1. Extensive investigations have shown that CUGBP1 activity is increased in DM cells, and results in a trans-dominant effect on gene splicing.

Although there is very good understanding of the consequences of the genetic mutation that causes DM1, the exact mechanisms responsible for the DM1 phenotype is not completely understood. DM1 pathogenesis is very complex and therefore, different potential approaches and multiple targets can be used for the development of DM1 therapies. To date, several attempts have been described as potential approaches for the therapy of the disease, either by restoring the levels of CUGBP1 and MBNL proteins, by targeting the mutant DMPK 3′ UTR transcripts or by the export of this toxic RNA from the nucleus to the cytoplasm of DM1 cells. All of the above approaches hold a great promise for the future

treatment of myotonic dystrophy, nevertheless it is also certain that more therapeutic approaches will be unveiled in the near future, which may be variations of the existing methods or novel ways to tackle the pathogenesis of the disease. Further research in DM1 needs to be done in order to move these therapies forward into clinical trials for the cure of DM1 disease.

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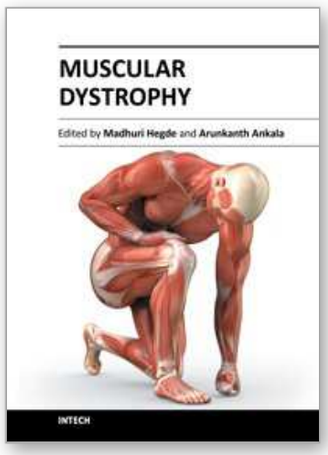

Muscular Dystrophy

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With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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