

# Antisense Oligonucleotides: Rising Stars in Eliminating RNA Toxicity in Myotonic Dystrophy

Zhihua Gao<sup>1</sup> and Thomas A. Cooper<sup>1-3</sup>

## Abstract

Myotonic dystrophy (DM) is a dominantly inherited, multisystemic disease caused by expanded CTG (type 1, DM1) or CCTG (type 2, DM2) repeats in untranslated regions of the mutated genes. Pathogenesis results from expression of RNAs from the mutated alleles that are toxic because of the expanded CUG or CCUG repeats. Increased understanding of the repeat-containing RNA (C/CUG<sup>exp</sup> RNA)-induced toxicity has led to the development of multiple strategies targeting the toxic RNA. Among these approaches, antisense oligonucleotides (ASOs) have demonstrated high potency in reversing the RNA toxicity in both cultured DM1 cells and DM1 animal models, thus offering great promise for the potential treatment of DM1. ASO targeting approaches will also provide avenues for the treatment of other repeat RNA-mediated diseases.

## Introduction

RNA-MEDIATED PATHOGENESIS is emerging as an important pathological mechanism in a wide range of diseases, particularly in microsatellite expansion disorders (Wheeler and Thornton, 2007; Cooper *et al.*, 2009). A growing number of autosomal dominant diseases, such as myotonic dystrophy (dystrophia myotonica; DM) (Brook *et al.*, 1992; Harley *et al.*, 1992; Mahadevan *et al.*, 1992), spinocerebellar ataxia (types 8, 10, and 12) (Koob *et al.*, 1999; Matsuura *et al.*, 2000), Huntington's disease-like 2 (HDL2) (Stevanin *et al.*, 2003; Rudnicki *et al.*, 2007), fragile X-associated tremor ataxia syndrome (FXTAS) (Aziz *et al.*, 2003; Hagerman *et al.*, 2004), and frontotemporal dementia/amyotrophic lateral sclerosis (FTD/ALS) (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011), have been found to be caused by mutations composed of expanded microsatellite repeats. Unlike the majority of inherited diseases caused by gene mutations in the protein-coding regions, however, several of the microsatellite expansion disorders are caused by expansion within noncoding regions, including introns, 5' untranslated regions (UTRs), and 3' UTRs (Ranum and Day, 2002; Cooper *et al.*, 2009). How mutations that do not affect the protein-coding region of genes cause an autosomal dominant disease has been a conundrum. However, a large number of studies have revealed a critical role for the toxic gain-of-function of repeat-containing RNA, independent of the encoded protein, as a predominant pathogenic factor in several of these microsatellite expansion diseases.

The first disease demonstrated to be caused by RNA-mediated toxicity was DM (Wheeler and Thornton, 2007). DM is a dominantly inherited, multisystemic disorder caused by expanded CTG repeats in the 3' UTR of the *DMPK* gene (type 1, DM1) or expanded CCTG repeats in the first intron of the *CNBP* gene (type 2, DM2) (Brook *et al.*, 1992; Mahadevan *et al.*, 1992; Liquori *et al.*, 2001). DM is the most common adult-onset muscular dystrophy and the second most common cause of muscular dystrophy after Duchenne muscular dystrophy (DMD), affecting approximately 1 of 8000 people worldwide (Mahadevan *et al.*, 1992; Day and Ranum, 2005). DM is characterized by progressive skeletal muscle weakness, myotonia, insulin resistance, cardiac arrhythmia, smooth muscle dysfunction, and neurological abnormalities (Harper, 2001).

The two types of DM are caused by different microsatellite expansions within different genes but share common (as well as different) clinical features (Harper, 2001; Udd and Krahe, 2012). DM1 is generally the more severe form. Unaffected individuals contain CTG repeat lengths of <38 units in the *DMPK* gene, whereas individuals with DM1 contain repeat lengths of 50 to >2000 units. The length of repeats directly correlates with the age of onset and the severity of disease (Harper, 2001).

To date, there is no effective treatment for DM. However, increased understanding of the repeat RNA-induced toxicity in DM pathogenesis has led to the rapid development of therapeutic strategies aimed at neutralizing or degrading the toxic RNA. These therapeutic approaches include antisense

<sup>1</sup>Department of Pathology and Immunology, <sup>2</sup>Department of Molecular and Cellular Biology, and <sup>3</sup>Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030.

oligonucleotides (ASOs), small interfering RNAs (siRNAs), small molecules, ribozymes, and engineered small nuclear RNAs (snRNAs) (Furling *et al.*, 2003; Mulders *et al.*, 2009; Pushechnikov *et al.*, 2009; Warf *et al.*, 2009; Wheeler *et al.*, 2009; François *et al.*, 2011; Childs-Disney *et al.*, 2012). Among these approaches, ASOs have demonstrated particularly promising results in removing CUG<sup>exp</sup> RNA, reversing downstream toxic consequences and suppressing the expansion of CTG repeats (Mulders *et al.*, 2009; Wheeler *et al.*, 2009; Nakamori *et al.*, 2011). Here we review advances in DM pathogenesis and therapeutic approaches with a major focus on the application of ASOs in DM1.

## DM Pathogenesis

Several hypotheses were proposed to explain the complex clinical features of DM1, including the following: (1) myotonic dystrophy protein kinase (dystrophia myotonia-protein kinase; DMPK) haploinsufficiency (Fu *et al.*, 1993; Hofmann-Radvanyi and Junien, 1993), (2) haploinsufficiency of neighboring genes (Klesert *et al.*, 1997; Thornton *et al.*, 1997), and (3) mutant DMPK RNA toxicity (Wang *et al.*, 1995; Caskey *et al.*, 1996). Early studies found reduced levels of DMPK mRNA and protein in DM1 patient samples, leading to the hypothesis that DMPK haploinsufficiency accounted for DM1 (Fu *et al.*, 1993; Hofmann-Radvanyi and Junien, 1993; Novelli *et al.*, 1993). In addition, because the CTG repeat expansion overlaps with the promoter region of the downstream *SIX5* gene and increased CTG repeat length correlated with reduced levels of Six5, *SIX5* haploinsufficiency was also proposed to cause DM1 (Klesert *et al.*, 1997; Thornton *et al.*, 1997). However, genetic inactivation of *DMPK* or *SIX5* in mice failed to produce prominent features of DM1 (Jansen *et al.*, 1996; Klesert *et al.*, 2000; Sarkar *et al.*, 2000), suggesting that altered expression of *DMPK* or *SIX5* does not contribute to the predominant aspects of the disease. Accumulating evidence has established a role for RNA-induced toxicity in DM1 pathogenesis. First, the similarity between the disease features of DM1 (CTG<sup>exp</sup>) and DM2 (CCTG<sup>exp</sup>) is consistent with a predominant role for an RNA gain-of-function in DM pathogenesis. DM1 and DM2 repeat expansion mutations occur at two distinct loci with no apparent functional links, suggesting that genes harboring these mutations are unlikely to be directly responsible for the disease. Second, transgenic mice (called HSA<sup>LR</sup>) expressing 250 repeats within the 3' UTR of an unrelated gene (human skeletal  $\alpha$ -actin, HSA) recapitulate critical features of DM1 in skeletal muscle, including myotonia and myopathy (Mankodi *et al.*, 2000). Mice expressing the same transgenic RNA but with only five CUG repeats exhibited no myopathy. Third, induced expression of CUG<sup>exp</sup> RNA in skeletal muscle and heart reproduces muscle weakness and heart conduction abnormalities (Mahadevan *et al.*, 2006; Wang *et al.*, 2007; Orengo *et al.*, 2008), establishing a prominent role for RNA-induced toxicity in DM1 pathogenesis. Further studies demonstrated that the expanded repeats (CUG<sup>exp</sup> or CCUG<sup>exp</sup>) cause the transcribed RNA to aggregate into nuclear foci readily detectable by *in situ* hybridization (Davis *et al.*, 1997). Results from several laboratories have shown that CUG<sup>exp</sup> or CCUG<sup>exp</sup> RNA induces cellular toxicity by disrupting the function of RNA-binding proteins, activating downstream signaling pathways or other mechanisms,

thereby playing a *trans*-dominant role in DM pathogenesis (Ranum and Day, 2004; Udd and Krahe, 2012).

### CUG<sup>exp</sup> RNA-induced toxicity

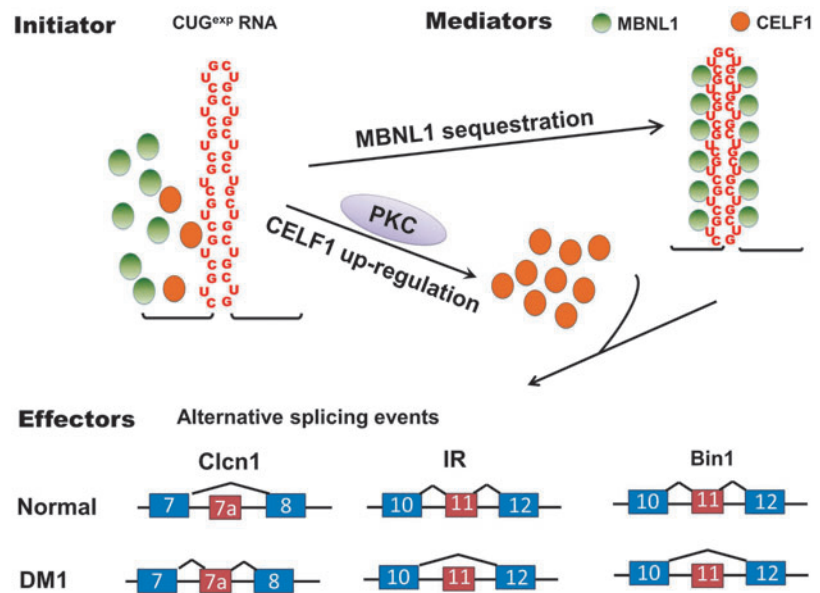
The RNA gain-of-function in DM has been characterized predominantly for CUG<sup>exp</sup> RNA in DM1. At least two mechanisms account for the repeat RNA-induced *trans*-dominant toxic effects. First, the CUG<sup>exp</sup> RNA forms a double-stranded hairpin structure that directly binds to the muscleblind-like (MBNL) family of proteins with high affinity, resulting in MBNL sequestration and loss-of-function (Miller *et al.*, 2000; Mankodi *et al.*, 2001). Second, CUG<sup>exp</sup> RNA activates protein kinase C (PKC) and upregulates CELF1 (CUGBP1 and ETR-3-like factor) protein levels, resulting in its gain-of-function (Kuyumcu-Martinez *et al.*, 2007; Wang *et al.*, 2007). MBNL and CELF proteins are antagonistic splicing factors that regulate alternative splicing events during development (Ho *et al.*, 2004; Lin *et al.*, 2006; Kalsotra *et al.*, 2008). Disruption of their functions by CUG<sup>exp</sup> RNA leads to widespread splicing dysregulation, in which embryonic splicing patterns are expressed; several of these misregulated splicing events contribute to disease features (Fig. 1). CELF and MBNL proteins also regulate RNA processing in addition to splicing that is likely to be relevant to DM pathogenesis (Dasgupta and Ladd, 2012; Wang *et al.*, 2012).

### Loss-of-function of MBNL

Humans and mice express three MBNL paralogues: MBNL1, -2, and -3. MBNL1 was first identified on the basis of its affinity for CUG<sup>exp</sup> RNA and colocalization with CUG<sup>exp</sup> RNA foci (Miller *et al.*, 2000; Mankodi *et al.*, 2001). Expression of CUG<sup>exp</sup> RNA results in sequestration and loss of MBNL1 function (Lin *et al.*, 2006). Genetic ablation of MBNL1 in mice results in extensive splicing defects in multiple tissues and reproduces critical features of DM1, including myotonia and cataracts (Kanadia *et al.*, 2003; Lin *et al.*, 2006; Du *et al.*, 2010; Suenaga *et al.*, 2012; Wang *et al.*, 2012). The majority of splicing defects observed in patients with DM1 were also detected in MBNL1 knockout mice (Kanadia *et al.*, 2003). Moreover, overexpressing MBNL1 in skeletal muscles of HSA<sup>LR</sup> mice expressing CUG<sup>exp</sup> RNA alleviates myotonia and reverses aberrant splicing (Kanadia *et al.*, 2006), establishing a critical role for MBNL1 loss-of-function in DM1.

Individuals affected by DM1 have variable levels of hypersomnia and cognitive and behavioral abnormalities (Harper, 2001; Weber *et al.*, 2010). MBNL1 knockout mice recapitulated important pathological features and aberrant splicing events of DM in skeletal muscle but exhibited only minor effects in brain (Kanadia *et al.*, 2003). Studies show that knockout of MBNL2, the more abundant MBNL paralogue in the nervous system, leads to extensive molecular and phenotypic defects in the mouse brain (Charizanis *et al.*, 2012). Notably, MBNL2 knockout mice develop hypersomnia associated with an increased propensity for rapid eye movement (REM) sleep, reminiscent of the DM1-related symptom. In addition, these mice exhibit decreased synaptic NMDA receptor activity, defective long-term potentiation, and impaired learning and memory (Charizanis *et al.*, 2012). Whether the widespread splicing changes directly contribute

**FIG. 1.** RNA-initiated toxicity in myotonic dystrophy type 1 (DM1) pathogenesis. The expanded CUG repeats in the myotonic dystrophy protein kinase (dystrophia myotonica-protein kinase; DMPK) 3' untranslated region (UTR) form a hairpin structure that binds to the splicing regulator muscleblind-like 1 (MBNL1) with high affinity, resulting in MBNL1 sequestration and loss-of-function. The CUG expansion (CUG<sup>exp</sup>) RNA also upregulates the levels of a second splicing factor, CUGBP Elav-like family member 1 (CELF1), by activation of protein kinase C (PKC). Disrupted function of MBNL1 and CELF1 leads to misregulation of a wide range of alternative splicing events including *Clcn1*, and *BIN1*, directly contributing to clinical features of DM1.



to brain abnormalities in mice and patients with DM1 remains to be determined. It is likely that CUG<sup>exp</sup> RNA-induced combinatorial loss-of-function of both MBNL1 and MBNL2 in different tissues extensively disrupts alternative splicing events implicated in DM1 pathogenesis.

#### Gain-of-function of CELF1

In addition to sequestration of MBNL1, CUG<sup>exp</sup> RNA also activates protein kinase C (PKC), leading to hyperphosphorylation of CELF1 protein, resulting in increased CELF1 stability and steady state levels (Kuyumcu-Martinez *et al.*, 2007; Wang *et al.*, 2007). Increased CELF1 protein levels were observed in DM1 skeletal muscle, heart, and myoblast cultures (Roberts *et al.*, 1997; Timchenko *et al.*, 2001; Dansithong *et al.*, 2005; Jones *et al.*, 2011). Elevated expression of CELF1 in skeletal muscle of transgenic mice produced muscle weakness and prominent central nuclei, a histological hallmark in DM1 (Ward *et al.*, 2010). Increased expression of CELF1 in mouse hearts resulted in left ventricular systolic dysfunction and dilation, as well as PR interval prolongation (Koshelev *et al.*, 2010), reproducing cardiac features of DM1. Induced expression of CELF1 in heart and muscle reproduced altered splicing events observed in DM1. Furthermore, CELF1 and MBNL1 play an antagonistic role in coregulating a subset of splicing, suggesting that the combined effects of MBNL1 loss-of-function and CELF1 gain-of-function contribute to DM pathogenesis (Ho *et al.*, 2004; Dansithong *et al.*, 2005).

#### Misregulation of alternative splicing

Misregulated splicing is a molecular hallmark of DM1 (Philips *et al.*, 1998; Savkur *et al.*, 2001; Charlet *et al.*, 2002; Mankodi *et al.*, 2003), with multiple aberrant splicing events caused by MBNL1 loss-of-function. For example, MBNL1 regulates the alternative splicing of a muscle-specific, voltage-dependent chloride channel, chloride channel-1 (*CLCN1*) (Kanadia *et al.*, 2003). In patients with DM, HSA<sup>LR</sup> mice, and MBNL1 knockout mice, aberrant inclusion of *Clcn1* exon 7a

introduces a premature termination codon (Charlet *et al.*, 2002; Mankodi *et al.*, 2002; Kanadia *et al.*, 2003), resulting in diminished expression of *Clc1* protein and myotonia (Lueck *et al.*, 2007). MBNL1 also promotes the inclusion of exon 11 of the insulin receptor (*IR*) (Ho *et al.*, 2004; Dansithong *et al.*, 2005). Inclusion of exon 11 produces an *IR* isoform (*IR-B*) with high signaling activity, whereas exclusion of exon 11 generates an isoform (*IR-A*) with increased affinity for insulin but low signaling activity. In patients with DM1, MBNL1 sequestration results in aberrant skipping of exon 11 in *IR* that is directly associated with insulin resistance (Savkur *et al.*, 2001). In addition, studies have shown that MBNL1 regulates alternative splicing of *BIN1*, a gene involved in T-tubule biosynthesis (Fugier *et al.*, 2011). Missplicing of *BIN1* in patients with DM1 leads to expression of an inactive form of *BIN1* that is unable to bind phosphatidylinositol 5-phosphate and to tubulate membrane, resulting in abnormal T tubule structures. Correction of abnormal *BIN1* splicing restores the sarcomere membrane structure and improves muscle contraction strength, implicating a role for *BIN1* missplicing in muscle weakness (Fugier *et al.*, 2011). However, MBNL1 knockout mice do not display overt muscle weakness or wasting, suggesting that additional mechanisms may be involved in the progressive muscle weakness in DM. One study shows that splicing of exon 29 in the CaV1.1 calcium channel, regulated by both MBNL1 and CELF1, is misregulated and may be associated with muscle weakness in DM (Tang *et al.*, 2012). Additional abnormal splicing events including cardiac troponin T (*cTNT*), sarcoplasmic/endoplasmic reticulum calcium ATPase-1 (*SERCA1*), Lim binding domain-3 (*LDB3*), and myomesin (*MYOM1*) have been observed in patients with DM and in mouse models (Philips *et al.*, 1998; Lin *et al.*, 2006; Hino *et al.*, 2007; Koebis *et al.*, 2011; Ohsawa *et al.*, 2011). However, the contribution of these misspliced events in DM pathogenesis is unclear.

#### Other mechanisms

In addition to MBNL1 and CELF1, RNA-binding proteins including heterogeneous nuclear ribonucleoprotein (hnRNP)

H and Staufen have also been implicated in DM pathogenesis (Kim *et al.*, 2005; Paul *et al.*, 2006; Mahadevan, 2012; Ravel-Chapuis *et al.*, 2012). Furthermore, antisense transcripts generated from the expanded repeats have been observed in DM1. For example, an antisense transcript adjacent to the *SIX5* regulatory region is produced and may be involved in local chromatin remodeling (Cho *et al.*, 2005). More recently, it has been shown that CUG<sup>exp</sup> RNA can also induce repeat-associated non-ATG-initiated (RAN) translation that may be involved in DM1 pathogenesis (Zu *et al.*, 2011). There are likely to be multiple mechanisms for DM pathogenesis; however, expression of CUG<sup>exp</sup> RNA is likely to be a predominant factor, making the RNA the major target of current therapeutic approaches.

### Therapeutic Strategies in DM1

Other than supportive care, no effective treatment is available for DM1. Several strategies have been developed to reduce the effects of DM1 pathogenesis, based on addressing the effects on CELF or MBNL. For example, overexpression of MBNL1 in HSA<sup>LR</sup> mice reversed the myotonia and missplicing events (Kanadia *et al.*, 2006; Chamberlain and Ranum, 2012). Inhibition of PKC activity in a heart-specific DM1 model alleviated the CUG<sup>exp</sup> RNA-induced cardiac conduction defects and partially reversed CELF1-regulated missplicing events (Wang *et al.*, 2009). In an approach targeting misregulated splicing of a specific gene product, restoration of normal *Clc1* splicing in HSA<sup>LR</sup> mice directly reversed the myotonia (Wheeler *et al.*, 2007). Although these approaches exhibited clear efficacy, they target specific consequences of the toxic RNA, leaving other pathogenic aspects intact. The most effective approach to eliminate the RNA-induced toxicity is to remove the toxic RNA. In support of this idea, reducing the expression of a tetracycline-inducible transgene encoding the human *DMPK* 3' UTR with five CTG repeats efficiently reversed the myotonia and cardiac defects caused by overexpression of the transgene (Mahadevan *et al.*, 2006), clearly demonstrating that removal of the pathogenic RNA can be beneficial to DM1.

To directly neutralize or remove the toxic RNA, various approaches including RNA interference (RNAi), ribozyme, small molecules, peptides, and ASOs have been developed (Langlois *et al.*, 2003, 2005; Mulders *et al.*, 2009; Warf *et al.*, 2009; Wheeler *et al.*, 2009, 2012; Garcia-Lopez *et al.*, 2011; Childs-Disney *et al.*, 2012; Lee *et al.*, 2012; Parkesh *et al.*, 2012). Among these approaches, ASOs have demonstrated particularly promising results in reversing the RNA toxicity, including restoration of normal activities of the RNA-binding proteins affected by CUG<sup>exp</sup> RNA, correction of missplicing events, and alleviation of myotonia. Here we focus on advances applying ASOs to target the toxic RNA in both DM1 patient cells and animal models.

#### A brief overview of ASO technology

ASOs target the RNA through base pairing to complementary nucleotide sequences. Natural nucleotides are not suitable for therapeutic application because of their sensitivity to cellular nucleases. Various chemical modifications have significantly improved the stability and binding affinity of ASOs such as phosphorothioate (PS), locked nucleic acid (LNA), 2'-*O*-methoxyethyl (MOE), 2'-*O*-methyl (OME),

peptide nucleic acid (PNA), and phosphorodiamidate morpholino (PMO) (Kole *et al.*, 2012). Of note, among these modifications, only the PS backbone modification is compatible with endogenous RNase H activity that specifically cleaves the RNA moiety in a DNA–RNA duplex (Lima *et al.*, 2004).

ASOs function via two general mechanisms: steric blocking or targeting enzymatic cleavage by RNase H. Steric-blocking ASOs are typically uniformly modified (MOE, OMe, LNA, or PMO) ASOs that bind to the RNA and prevent the binding of factors (proteins or RNAs) without inducing RNA degradation (Bennett and Swayze, 2010). For example, ASOs specifically designed to block splice sites at intron–exon junctions or *cis*-acting splicing regulatory elements have been used to redirect the splicing of dystrophin in DMD and *SMN2* in spinal muscular atrophy (SMA) and are currently being used in clinical trials (van Deutekom *et al.*, 2007; Kinali *et al.*, 2009; Hua *et al.*, 2010, 2011; Muntoni and Wood, 2011). In contrast, ASOs competent for RNase H-mediated degradation are typically designed as “gapmers” containing 6–10 central nucleotides with RNase H-competent PS modifications flanked by 3 or 4 nucleotides at the 5' and 3' ends that contain modifications that further stabilize the ASO but are not RNase H competent (Bennett and Swayze, 2010). On binding to the target RNA, the “gap” in the gapmer–RNA duplex allows RNase H-mediated cleavage, resulting in specific degradation of RNA. Two gapmers are currently in clinical trials: (1) mipomersen, a gapmer targeting the apolipoprotein B100 mRNA, is used to reduce the level of low-density lipoprotein cholesterol in patients with familial hypercholesterolemia (Raal *et al.*, 2010); and (2) OGX-11, a gapmer targeting the clusterin mRNA, is used to induce cancer cell apoptosis in patients with metastatic prostate cancer (Chi *et al.*, 2010).

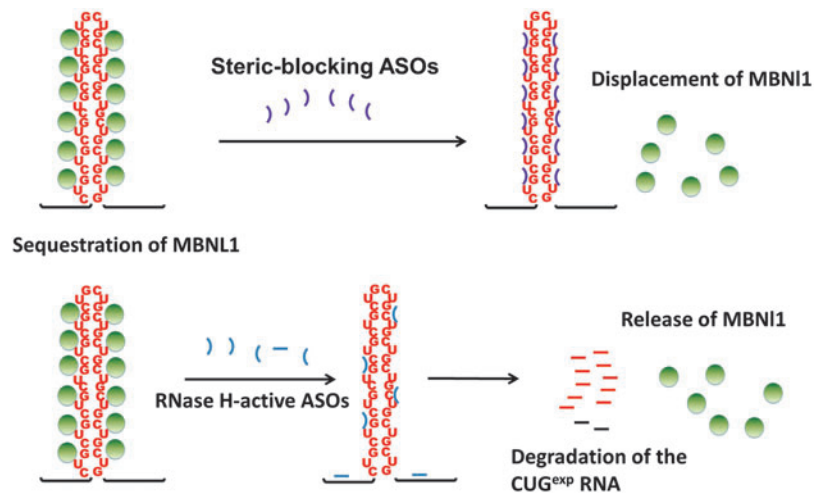
#### Application of ASOs to target toxic CUG<sup>exp</sup> RNA in DM1

ASOs delivered to cells go to the nucleus (Leonetti *et al.*, 1991), making them attractive reagents in targeting nuclear transcripts such as CUG<sup>exp</sup> RNA. Both steric-blocking ASOs and gapmers have been tested in DM1 patient cells and mouse models to target the CUG<sup>exp</sup> RNA with high specificity and efficacy, demonstrating great potential for future DM1 treatment (Fig. 2).

In 2009, two separate studies showed that steric-blocking ASOs can disrupt the CUG<sup>exp</sup> RNA–MBNL1 interactions and reverse RNA-induced toxicity in mouse models and DM1 myoblasts. Using a 25-nucleotide CAG morpholino (CAG25) that binds to CUG<sup>exp</sup> RNA with high affinity, Wheeler and colleagues (2009) demonstrated that CAG25 effectively prevented the formation of the RNA–MBNL1 complex and displaced MBNL1 from the preformed RNA–protein complex *in vitro* (Wheeler *et al.*, 2009). When applied *in vivo* to the skeletal muscle of HSA<sup>LR</sup> transgenic mice, a single intramuscular injection of CAG25 dissolved the RNA ribonuclear foci, resulting in the release of MBNL1, reversal of myotonia, and restoration of normal splicing transitions for at least 14 weeks (Wheeler *et al.*, 2009).

In an independent study, Mulders and colleagues screened 2'-*O*-methylphosphorothioate ASOs that annealed along the *DMPK* mRNA including the CUG repeat region and

**FIG. 2.** Application of antisense oligonucleotides (ASOs) to eliminate RNA toxicity in myotonic dystrophy type 1 (DM1). Steric blocking ASOs bind to CUG<sup>exp</sup> RNA with high affinity and disrupt the RNA–MBNL1 (muscleblind-like 1) interactions, thereby displacing MBNL1 from the toxic RNA and reversing some features of DM1. In contrast, RNase H-active ASOs bind to the CUG<sup>exp</sup> RNA and directly induce the degradation of the toxic RNA, resulting in release of MBNL1, restoration of its normal function, and reversal of disease features.



found that PS58, a (CAG)<sub>7</sub> oligonucleotide complementary to the CUG repeats, could effectively induce degradation of CUG<sup>exp</sup> RNA in DM1 myoblasts and mouse models (Mulders *et al.*, 2009). Although wild-type DMPK mRNA contains CUG repeats, both CAG25 and PS58 appeared to specifically target the mutant CUG<sup>exp</sup> RNA, possibly due to the high binding capacities of the expanded repeats. Intriguingly, ASOs used in both studies significantly reduced levels of CUG<sup>exp</sup> RNA (50% for CAG25 PMO, 80% for PS58) even though the ASOs were not designed to induce enzymatic cleavage of the target RNA (Mulders *et al.*, 2009; Wheeler *et al.*, 2009). The breakdown of the RNA–MBNL1 complex by ASOs facilitated the export of RNA to the cytoplasm (Wheeler *et al.*, 2009), suggesting the RNA is made available for degradation machinery in the nucleus or cytoplasm. However, the released CUG<sup>exp</sup> RNA from the ribonuclear foci also raise the possibility that CUG<sup>exp</sup> RNA, free of MBNL1 binding, can induce cellular toxicity through MBNL1-independent mechanisms (Cooper, 2009; Mahadevan, 2012).

The most attractive approach to eliminate RNA toxicity is to directly degrade the toxic RNA. RNase H activity removes the RNA primer for DNA synthesis on Okazaki fragments during DNA replication. The nuclear localization and essentially ubiquitous expression of RNase H (Suzuki *et al.*, 2010) make RNase H-active ASOs an ideal tool with which to target nuclear-retained CUG<sup>exp</sup> RNA in the cell types affected in DM1. Two studies have demonstrated the high efficacy and potency of using RNase H-active ASOs in targeting the CUG<sup>exp</sup> RNA in both DM1 cell culture and mouse models. Using gapmers that contain CAG sequences complementary to the CUG repeats, Lee and colleagues showed that LNA- or MOE-flanked gapmers can effectively reduce the levels of toxic RNA and disrupt RNA foci in a cell culture model of DM1 (Lee *et al.*, 2012). These effects were highly specific to expanded CUG transcripts, as repeats of normal length were not significantly affected, suggesting selective gapmer targeting of the mutant allele. When administered to the skeletal muscle of a DM1 mouse model by direct injection, gapmers targeting the CUG repeats also showed efficacy in removing the CUG<sup>exp</sup> RNA. However, injected ASOs induced histological abnormalities in skeletal muscle (Lee *et al.*, 2012). It is possible that the toxicity is due to the combination of the ASO and an inflammatory response

induced by intramuscular injection. If this is the case, systemic administration is expected to produce less toxicity.

Combined administration of gapmers (RNase H-active) and the CAG25 morpholino (RNase H-inactive) was used to test whether disruption of the RNA foci with CAG25 would increase availability of CUG<sup>exp</sup> RNA to degradation by the CAG gapmer. In both DM1 cell culture and skeletal muscle of DM1 mouse models, combined administration produced synergistic effects, suggesting that combined application of ASOs through different pathways may help to maximize therapeutic efficacy. These studies showed limited effects, however, because of competition between gapmer and morpholino ASOs both targeting the CUG repeats. An improved strategy would use CAG25 to disrupt RNA–protein interactions and gapmer ASOs to target another region of the DMPK mRNA.

In the experiments described previously, ASOs were delivered locally to a single muscle by intramuscular injection. Whether systemic application of ASOs is useful in removing the toxic CUG<sup>exp</sup> RNA was unclear, because of the poor intracellular uptake of ASOs in some tissues, particularly those most affected in DM1: skeletal muscle, heart, and brain (Muntoni and Wood, 2011). Notably, a robust reduction of toxic RNA in skeletal muscles was observed after subcutaneous injection of gapmers that target the transgenic HSA mRNA containing 250 CUG repeats in HSA<sup>LR</sup> mice (Wheeler *et al.*, 2012). The reduced level of CUG<sup>exp</sup> RNA was accompanied by loss of nuclear RNA foci, release of MBNL1, correction of myotonia, and restoration of misregulated splicing, with no apparent off-target effects (Wheeler *et al.*, 2012). The most astonishing effect was that the silencing of the CUG<sup>exp</sup> RNA was sustained for 1 year after the treatment was terminated. The persistent effects appear to be specific to nuclear-retained RNAs, as ASOs targeting Pten mRNA that is rapidly transported to the cytoplasm were ineffective (Wheeler *et al.*, 2012). RNase H-active ASOs thus provide clear advantages to effectively target the nuclear-trapped CUG<sup>exp</sup> RNA for potential therapeutic applications.

### Perspective and Conclusions

ASOs are a promising approach to eliminate RNA toxicity; however, challenges remain for large-scale therapeutic

application in DM1. First, DM1 is a multisystemic disease and therefore requires efficient delivery of ASOs to multiple tissues. Although systemic delivery effectively removed CUG<sup>exp</sup> RNA, reversed the splicing events, and alleviated myotonia in skeletal muscle, efficient gapmer delivery to heart and brain is more challenging (Bennett and Swayze, 2010; Muntoni and Wood, 2011). Second, with the high potency of gapmers comes the potential for off-target effects (Bennett and Swayze, 2010). Although no apparent off-target effects were observed in animal experiments with the ASOs targeting human ACAT1 in the HSA<sup>LR</sup> transcript (Wheeler *et al.*, 2012), a therapeutic ASO will target human DMPK. Because the entire DMPK mRNA is the target, there are likely to be multiple ASOs that will induce efficient mRNA degradation that can then be screened for minimal off-target effects. In addition to targeting the “wrong” mRNA, ASOs may also have aptameric-like properties by interacting with certain proteins (Marques and Williams, 2005). The long-term pharmacokinetics and potential for toxicity of gapmers need to be fully explored before their large-scale clinical application. Third, there are concerns regarding the potential for reduced expression of DMPK protein. The nuclear localization of both RNase H and the toxic CUG<sup>exp</sup> RNA leads to preferential degradation of RNA from the expanded allele. However, there is likely to be some level of degradation of mRNA from the normal allele. Given that RNA from the expanded allele is sequestered, the mutation produces haploinsufficiency of DMPK. Degradation of RNA from the mutant allele will not add to this deficiency and will be beneficial by removing the toxic RNA. However, degradation of mRNA from the normal allele will further decrease DMPK protein expression. In DMPK knockout mice, there is little effect of hemizygosity but complete loss of DMPK function produced cardiac abnormalities in older animals (Jansen *et al.*, 1996). Therefore, the effects on the wild-type allele will need to be carefully monitored.

In parallel with the rapid development of ASOs, other strategies including screening of small chemicals and peptides that specifically bind to CUG<sup>exp</sup> RNA have also made promising progress (Warf *et al.*, 2009; Garcia-Lopez *et al.*, 2011; Childs-Disney *et al.*, 2012). The strategy for most small-molecule approaches is to alter RNA–protein interactions potentially making CUG<sup>exp</sup> RNA more soluble. Combinatorial approaches using ASOs with small molecules could lead to effective CUG<sup>exp</sup> RNA degradation with reduced doses of both reagents. Chemicals such as the peptide ABP1 and rationally designed small molecules that bind to CUG<sup>exp</sup> RNA with high affinity would be good candidates for further pharmaceutical and therapeutic development (Garcia-Lopez *et al.*, 2011; Childs-Disney *et al.*, 2012). In addition, linking ASOs with peptides or nanoparticles may significantly improve drug delivery to DM-affected tissues such as heart (Yin *et al.*, 2011; Betts *et al.*, 2012).

The understanding of pathogenic mechanisms of DM has progressed rapidly in the 20 years since the identification of the DM1 disease gene in 1992. Although many details of the pathogenic mechanism remain to be delineated, the identification of the crucial role of the toxic RNA has led to the rapid development of therapeutic strategies in DM1. The efficacy, potency, and high selectivity of ASOs in targeting the pathogenic RNA offer the promise for potential treatment

in DM1. Successful development of RNA-targeting therapeutic strategy in DM1 will also offer new opportunities for treatment of other RNA-dominant diseases caused by microsatellite expansion.

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### Author Disclosure Statement

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Address correspondence to:

Dr. Thomas A. Cooper

Department of Pathology and Immunology

Baylor College of Medicine

Houston, TX 77030

E-mail: tcooper@bcm.edu

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