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REVIEW

Progress in therapeutic antisense applications for neuromuscular disorders

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Neuromuscular disorders are a frequent cause of chronic disability in man. They often result from mutations in single genes and are thus, in principle, well suited for gene therapy. However, the tissues involved (muscle and the central nervous system) are post-mitotic, which poses a challenge for most viral vectors. In some cases, alternative approaches may use small molecules, for example, antisense oligonucleotides (AONs). These do not deliver a new gene, but rather modulate existing gene products or alter the utilization of pathways. For Duchenne muscular dystrophy, this approach is in early phase clinical trials, and for two other common neuromuscular disorders (spinal muscular atrophy and myotonic dystrophy), significant preclinical advances have recently been made.
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INTRODUCTION

Neuromuscular disorders (NMDs) are a frequent cause of loss of ambulation, chronic disability, and early death worldwide. An estimated 300 000 people suffer from NMDs in Europe alone. Some of these disorders have a genetic cause, with mutations in different genes leading to a variety of phenotypes that differ in severity and/or affected muscles.[1] As yet, treatment is largely palliative and delays, but does not prevent the progressive loss of muscle and/or function and increasing disability. In principle, gene therapy to replace the defective gene is an attractive approach.[2] However, whole body treatment is challenging for NMDs because of the tissues involved: ~30 to 40% of the human body consists of muscle, and delivery to the central nervous system is impeded by the blood–brain barrier. Although adeno-associated virus (AAV) is one of the few viral vector systems that efficiently infects muscle, it has a small cloning capacity.[3] Not only do cDNAs of most NMD genes exceed this capacity, but also many genes are regulated by long and complex promoters, involving multiple start sites and complex alternative splicing.

For some NMDs (eg, myotonic dystrophy (DM)), a dominant-negative effect underlies the disease. For these, replacing the defective gene is unlikely to be therapeutic at all. Thus, it is not surprising that for NMDs, research also focuses on ways to restore gene expression at the (pre-) mRNA level. This can be achieved through antisense oligonucleotides (AONs), small synthetic RNAs, DNAs or analogs, which hybridize specifically to their target sequences.[2] Today, AONs are in clinical trials for several applications (Table 1). They are relatively small (~10 kDa) and have more favorable biodistribution properties than, for example, plasmids.[4] They can be produced on a large scale under GMP conditions much easier than viral vectors. For three of the major NMDs (Duchenne muscular dystrophy (DMD), Spinal muscular atrophy (SMA) and Myotonic dystrophy), AONs have therapeutic potential and are currently in various stages of translational trajectories.[5–8] Interestingly, although the tool is the same for each disease (AONs), it is tailored in different ways for the different NMDs (see below). For DMD, AONs are already in Phase IIa clinical trials and are generally considered the most promising therapy for this disease.[7,9] For SMA and DM, studies are only preclinical so far, but major advances have been made in the recent past[5,6,8,10] and clinical trials are likely to take place in the near future.

Here, we will review the different therapeutic approaches for DMD, SMA and DM, and discuss the development toward clinical application of these treatments.

DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is the most common childhood NMD. The disease is inherited X-recessive and has an incidence of 1 in 3500 newborn boys. DMD is caused by mutations in the *DMD* gene that result in non-functional dystrophin proteins.[11,12] Dystrophin is required for muscle fiber membrane stability on exercise, and consequently, loss of functional dystrophin leads to recurrent muscle fiber damage during contraction. After initial attempts at regeneration, muscle fibers are eventually replaced by adipose and fibrotic tissue. This process is accompanied by the progressive loss of muscle function and generally leads to wheelchair dependency before the age of 13 and premature death, mostly before the age of 30.[1] DMD treatment is only symptomatic, but corticosteroids and assisted ventilation have significantly increased the quality of life and the life expectancy of patients during the last few decades.[13,14]

Interestingly, there is an allelic disease, Becker muscular dystrophy (BMD), in which mutations in the same gene maintain the open reading frame and allow the production of a partially functional dystrophin protein.[1,12,15] BMD patients have a varying phenotype, but are less severely affected; the disease progression is typically much slower and they have a longer to normal life expectancy.[1]

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Table 1 Overview of antisense oligonucleotide applications

Mechanism	*Application*	*Stage*	*References*
RNase H knockdown of DNA-RNA hybrids	Antiviral therapy Anticancer therapy	Registered drug phase III clinical trials	95,96
Immune activation through toll-like receptor 9	Anticancer therapy Vaccination adjuvant	Phase II clinical trials	97
Translation initiation/elongation block	Therapy (anticancer, antiviral, immune modulation, hypercholesterolemia) Developmental studies (mainly zebrafish)	Phase III trials	98,99
Splice modulation	Prevent cryptic splicing Modulate alternative splicing Restore open reading frame	Phase II clinical trials	7
Gene correction through mismatch repair mechanisms	Correct small mutations	Preclinical, low efficiency	100

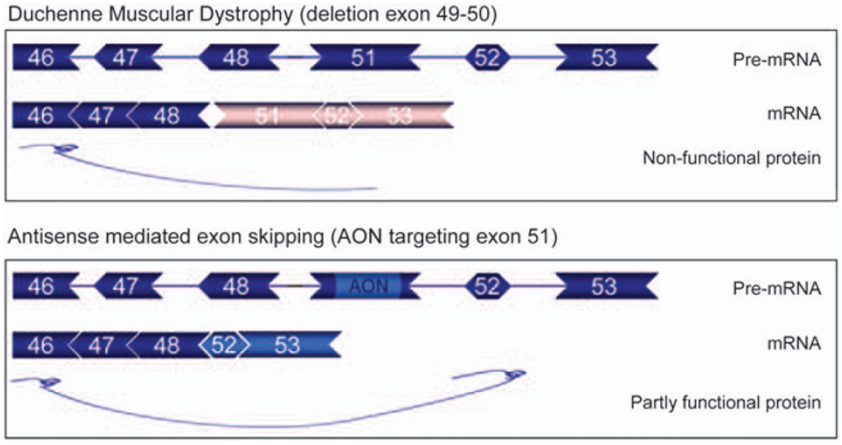

Figure 1 Antisense-mediated exon skipping for DMD. DMD is caused by mutations (in this example, a deletion of exons 49–50, upper panel) in the *DMD* gene that disrupt the open reading frame. Consequently, protein translation is truncated prematurely and the resulting dystrophin cannot fulfill its normal function (ie, to connect the extracellular matrix and the cytoskeleton in muscle fibers). AONs complementary to an exon (exon 51 in this example, lower panel) can hide this exon from the splicing machinery during pre-mRNA splicing, resulting in the skipping of the targeted exon. By strategically targeting specific exons in DMD patients, the reading frame can be restored through exon skipping, allowing the synthesis of an internally truncated protein that is partially functional.

Antisense-mediated reading frame restoration approach

AONs can be used to reframe dystrophin transcripts, by hiding an exon from the splicing machinery and causing the skipping of said exon (Figure 1).[2] This will allow the generation of BMD-type, internally deleted, partly functional dystrophin proteins and should minimally convert a severe into a milder phenotype. Proof of concept has been obtained in patient-derived tissue cultures and mouse models carrying different (types of) mutations. For deletions of one or more exons (present in 65% of all DMD patients), the skipping of one or two additional exons resulted in reading frame and dystrophin restoration for the majority of treated myogenic cells.[16–19] Typically, small mutations (28% of all DMD mutations) can be bypassed by skipping the exon harboring the mutation, provided the mutation is not present in the first or the final (79th) exon, as these cannot be skipped. For in-frame exons – there are 39 exons for which the number of nucleotides is divisible by three – this is sufficient.[20] For out-of-frame exons, the reading frame has to be restored by skipping an adjacent exon, so that the total number of lost nucleotides becomes divisible by three. This double exon skipping is feasible for 31 of 38 out-of-frame exons.[20] For both mutations in in-frame and out-of-frame exons, exon skipping led to dystrophin re-expression in cultured myogenic cells[16,17,21] and two dystrophic mouse models (*mdx* and *mdx*^{4cv}), which carry point mutations in mouse exons 23 and 53, respectively.[22,23] Furthermore, a point mutation in a splice site causing exon 7 skipping in a dog model of the disease has been corrected by exon 6 and 8 skipping in cultured cells and *in vivo*[24,25] AONs have been used to restore normal splicing in very rare small intronic mutations that induce the inclusion of cryptic exons[26] and to restore the reading frame for a single patient with an inversion of exons 49 and 50.[27] For duplications (present in 7% of DMD patients[28]) exon skipping is more challenging, as the original and the duplicated exons are indistinguishable for the AONs. In case of single exon duplications, either exon can be skipped to restore the wild-type transcript. This has been achieved in cultured cells from a patient with an exon 45 duplication.[29] However, for other single exon duplications, exon skipping was so efficient that both exons were skipped, thus once more disrupting the open reading frame.[29,30] Through skipping of the duplicated exons as well as of an additional adjacent exon, the transcripts could be reframed.[29] For multiple exon duplications, exon skipping is more complex, as only the skipping of one or two specific duplicated exon(s) is beneficial, whereas the skipping of most other combinations of exons still disrupts the open reading frame.

The exon-skipping approach is fundamentally mutation-specific and, as such, a clear example of 'personalized medicine.'[9] In theory, exon skipping would be applicable to almost 80%[20] of all patients and AONs to induce the skipping of each dystrophin exon, except for the first and the last that have been identified.[31,32] As 70 and 25% of all deletions occur in the major (exons 45–53) and minor (exons 2–11) hotspots, respectively, and deletions are present in 65% of patients,[28] skipping of some exons is applicable to large groups of patients. The most notable example is exon 51 skipping, which is applicable to 13% of all patients (or 19% of all deletion patients).[20] Skipping of the 10 most applicable exons would already be applicable to 41% of all patients (or 60% of all deletion patients).[20]

Toward clinical application

The exon-skipping approach was first tested *in vivo* in the *mdx* mouse model. Intramuscular injections with ~5–20 μg AONs targeting exon[23] resulted in exon skipping and dystrophin restoration that persisted for up to 3 months and was accompanied by functional improvement.[23] In addition, proof of concept of this approach in man was recently delivered in a first-in-man clinical trial. Four DMD patients were injected locally in the *tibialis anterior* muscle with a single dose of 0.8 mg PRO051, targeting exon 51 and applying 2′-*O*-methyl phosphorothioate chemistry.[7] In each patient, dystrophin was restored in the vast majority of muscle fibers at levels varying between 17 and 35%, in the absence of treatment-related adverse events. A similar trial is currently ongoing in the United Kingdom with 0.09 and 0.9 mg of a phosphorodiamidate morpholino oligomer (PMO, AVI 4658) also targeting exon 51.[33]

Although the results of the first clinical trial and preliminary results of the second trials are encouraging, intramuscular injection of each individual muscle is not feasible. Therefore, ways to deliver AONs systemically are now under study. Several different AON chemistries, each with different properties regarding biodistribution and biokinetics, are currently under investigation. These chemistries are also used in clinical trials, and hence safety data (albeit rather limited) are available for humans.[4] Interestingly, biodistribution studies in mice have revealed that AONs are taken up more easily by dystrophic than healthy muscle. This is presumably because of the leakiness of the dystrophic fibers,[34] but is in fact one of the few examples where the disease state assists in therapy. Systemic treatment (intravenous, intra-arterial, intraperitoneal or subcutaneous injection) of ~100 mg/kg 2′-*O*-methyl phosphorothioate or PMO AONs resulted in body-wide exon skipping, dystrophin restoration and functional improvement[35–37] and (Heemskerk JA *et al* manuscript submitted). Following these encouraging results, dose-finding/safety Phase I/II trials are currently ongoing with PRO051 and AVI4658. These trials are essential not just to confirm safety and determine the optimal dose of the AONs, but also to determine as yet unknown parameters, such as the serum half-life of the respective AONs in humans.

It is noted that for both chemistries, mouse studies revealed that the exon skipping and dystrophin levels were much lower or even non-existent in the heart.[35–37] Direct injection in the ventricle wall resulted in local exon-skipping and dystrophin production, but at much lower levels than that observed after intramuscular injection in skeletal muscles.[38] This is probably because of the fact that although skeletal muscle fibers show damage and membrane tears, heart cells do not. Thus, although AONs can diffuse into muscle fibers through these holes, heart cells do not have this 'advantage.' Interestingly, conjugated cell-penetrating arginine-rich peptides to PMOs greatly improve uptake by skeletal muscles and also by the heart. This would allow lower doses of AONs, while resulting in higher dystrophin levels in skeletal and heart muscles, accompanied by lower serum creatine kinase levels and improved muscle and cardiac histology.[39–41] So far, mice have only been treated with these conjugated AONs for brief time periods, and therefore no efficacy and toxicity data have yet been published for longer treatment. However, if safety profiles are favorable, these conjugated AONs are very promising for systemic AON treatment.

Owing to AON and muscle fiber turnover, therapeutic AONs will have to be delivered repeatedly. To achieve more permanent exon skipping, an 'antisense gene' may be introduced with viral vectors. These antisense genes are usually modified U7 or U1 small nuclear ribonucleoprotein (snRNP) genes, with the original antisense sequences that hybridize to histones or the 5′ splice site, respectively, replaced by the AON antisense sequence.[42,43] As the snRNP genes are very small, they easily fit in the AAV vector. Indeed, long-term (18 months), body-wide exon skipping, dystrophin restoration and improved muscle morphology have been observed in the skeletal muscles and heart of *mdx* mice treated with AAV vectors containing modified snRNP genes.[42–44] However, although AAV appears to be non-immunogenic in mice, it is in dogs[45] and humans[46] which precludes repeated administration. Moreover, it is a major challenge to obtain the number of AAV vector particles required for whole body treatment of DMD patients at clinical grade.[47] By contrast, AONs can be produced under GMP conditions at a high scale relatively easily.

A key regulatory hurdle both for AONs and AAV-delivered antisense sequences is that presently each AON or antisense sequence is considered a new drug by the drug administration offices. Thus, AONs targeting different exons all have to go through extensive toxicity studies and clinical trials individually. This makes the development of different AONs expensive for all mutations, and close to impossible for the lower frequency mutations, as the number of patients required in clinical trials simply is not present worldwide.[48]

SPINAL MUSCULAR ATROPHY

Spinal muscular atrophy (SMA) is the second most common autosomal recessive disease with an incidence of ~1 in 6000.[49] The most severe form (SMA type I) is the leading cause of infantile death, and less severe forms (type II and III) are a major cause for loss of ambulance and/or chronic disability. The disease is caused by homozygous loss of the 'survival of motor neuron 1' (*SMN1*) gene.[50] This encodes an essential protein for the assembly of small nuclear ribonucleoproteins (snRNPs), the major components of the spliceosome.[51] As a consequence, α-motor neurons die in the anterior horn spinal cord, leading to a neurodegenerative disease characterized by the progressive paralysis of the trunk and limbs and pervasive, generalized weakness. Complete lack of SMN is embryonic lethal in mice.[52] However, in humans, low levels of SMN protein are generated by the adjacent, homologous *SMN2* gene, with opposite polarity.[49] As the disease manifests only in motor neurons, these low levels are apparently sufficient for normal function of all other cells. The low SMN2 level is attributed to a translationally silent point mutation in exon 7, disrupting an exonic splicing enhancer site, while inducing an exonic splicing silencer (see Figure 2 for an explanation about exonic and intronic splicing enhancers and silencers). Consequently, this exon is largely (90%) skipped, resulting in an unstable SMN protein.[53–55] Functional, full-length protein can only be generated from the minor amount of transcripts including exon 7. The *SMN2* gene is present in 1–5 copies in the population, resulting in 10–50% of full-length protein in patients. This explains the genetic heterogeneity of the disease phenotype for SMA type 1 (10% protein) to the less severe types 2 and 3 (20% or more full size protein).[56]

Antisense-mediated exon inclusion approach

As the number of *SMN2* copies correlates inversely with disease severity,[56] moderate increases in SMN protein levels may already have significant beneficial effects. Therefore, ways to increase exon inclusions are under investigation. A number of drugs that increase *SMN2* expression and/or enhance exon 7 inclusion have been identified, including sodium butyrate,[57] sodium vanadate,[58] aclarubicin[59] and valproic acid.[60] However, these drugs act nonspecifically, and so it is anticipated that they can also disrupt the splicing of other genes, leading to off-target effects. Obviously, AONs targeting SMN2 transcripts to induce exon 7 inclusion would be more specific. It has been postulated that exon 7 skipping occurs because the C to T transition disrupts an ESE,[61] introduces an ESS,[62] or both. However, exon 7 and flanking regions contain many additional enhancing and silencing elements that sometimes overlap partly (Figure 3).[63–68] The most straightforward approach is to use AONs to block exonic or intronic silencing sequences to enhance exon inclusion. These AONs have indeed been tested in *in vitro* splicing experiments,[69] although some AONs unexpectedly induce exon skipping, suggesting that exon 7 splicing is regulated by additional motives. This is not surprising as the exon 7 splice sites are very weak,[66] especially the acceptor (3′) splice site.[70] Thus, the much stronger acceptor splice site of exon 8 may compete and be selected more often over the exon 7 splice site. It has been feasible to enhance exon 7 inclusion using AONs blocking the exon 8 acceptor splice site.[70–72] As exon 8 is the final exon, and the stop codon is present in exon 7, transcripts containing exon 7, but not exon 8, include the complete SMN2 coding sequence giving rise to full-length SMN proteins.[72]

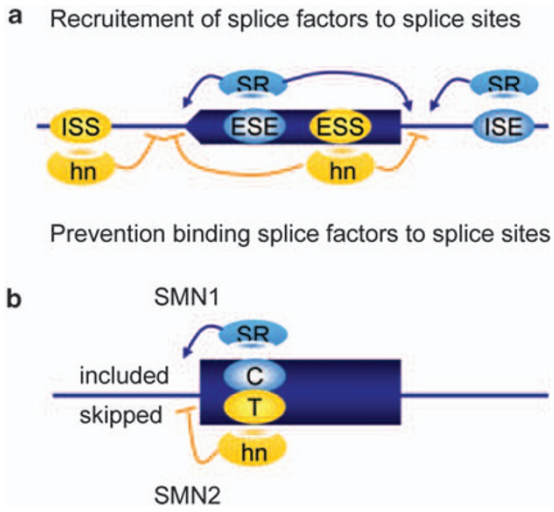

Figure 2 Exonic and intronic splicing enhancers and silencers. (**a**) Pre-mRNA splicing is a highly complex process that is orchestrated by the spliceosome, and involves hundreds of different proteins and small nuclear RNAs (snRNAs). The start and the end of introns are recognized by the splicing machinery as these so-called splice sites contain consensus sequences. However, not all exons have strong splice sites, and consensus-like sequences are also present in introns. A family of splicing factors facilitates the recognition of proper exons through binding to exonic/intronic splicing enhancer sequences (E/ISEs) and impedes the inclusion of pseudoexons by binding to intronic/exonic splicing silencers (I/ESSs). The splicing factors involved are SR proteins (eg, SF2/ASF and Tra-2β) and hnRNPs, respectively. Generally, splicing enhancers are located in exons, and silencers in introns, but this is not always the case. Some exons contain both enhancer and silencer elements, and, depending on the tissue-specific levels of SR and hnRNP proteins, the exon is included or excluded from the transcript. (**b**) In exon 7 of *SMN2*, a translationally silent mutation disrupts an ESE while it creates an ESS. As a consequence, the splicing enhancing SF2/ASF can no longer bind, whereas the splicing silencing hnRNP A protein can now bind. The exon is no longer properly recognized and skipped in most transcripts.

Recently, Krainer and co-workers performed systematic 'AON walks' in intron 6, exon 7 and intron 7 *in vitro* in a minigene to identify the most optimal AON for exon 7 inclusion.[5,10] Promising AONs were also tested in patient-derived cells and intravenously injected into an SMA mouse model that contains a copy of the human *SMN2* gene. This led to increased SMN protein levels in cells, in liver and to a lesser extent in kidney and muscle in the mouse model.[5,10] Unfortunately, no exon skipping or SMN protein increase has been observed in the central nervous system yet. It is known that AONs are unable to cross the blood–brain barrier, and so direct injection into the spinal cord or the central nervous system is required. Indeed, it has already been shown that intraventricular injections of AONs targeting the exon 8 splice site resulted in an increase in SMN production in the brain.[72]

Another way to shift splicing uses AONs that contain a tail with ESE motives. The rationale is that these AONs will induce exon inclusion, regardless of whether they target an ESE or an ESS sequence, as the tail will act as an enhancer and recruit the required splicing factors to the exons to facilitate exon inclusion. Proof-of-principle was obtained with a tailed AON that induced dose-dependent exon 7 inclusion in an *in vitro* splicing system[73] and in patient fibroblasts where SMN levels were increased accordingly.[74] Alternatively, infection with AAV or lentiviral vectors expressing bifunctional RNAs or inducible U7 snRNPs targeting exon 7 and simultaneously recruiting SR proteins enhanced exon 7 inclusion in patient-derived fibroblasts that was accompanied by increased SMN levels.[75–77] Further proof of concept for this approach has been presented recently with the generation of transgenic mouse models expressing bifunctional RNAs in an *SMN1*-negative background, but with a copy of the human *SMN2*

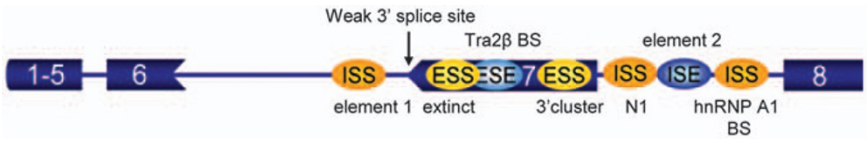

Figure 3 Splicing enhancers and silencers in and flanking *SMN2* exon 7. *SMN* exon 7 splicing is regulated by many enhancing (blue) and silencing (yellow) elements located in intron 6, the exon itself and intron 7, and the exon has a weak 3′ splice site. An intronic splicing silencer, called element 1, is located in intron 6. The C to T transition in the proximal part of the exon disrupts an exonic splicing enhancer that normally recruits SF2/ASF and creates a new splicing silencer that binds hnRNP A1 (extinct). A Tra-2β binding site is located downstream of the extinct in the so-called conserved region that acts as an ESE. An additional silencer is located at the end of the exon in the 3′ cluster. Two intronic splicing silencers are located in intron 7, interspersed by an intronic splicing enhancer. Together, these sequences make up 'element 2.' The first silencer is called 'N1.' The more recently discovered second silencer has been shown to bind to hnRNP A1.

gene (there is no *SMN2* homolog in the mouse). Transgenic expression of the antisense RNAs induced exon 7 inclusion, restored SMN production in motor neurons and complemented the lethal phenotype.[78]

MYOTONIC DYSTROPHY

Myotonic dystrophy (DM) is the most common adult onset NMD with an incidence of 1 in 8000 in Europe and North America and shows an autosomal dominant inheritance pattern.[79] The disease is characterized by neuromuscular symptoms including myotonia, progressive muscle weakness, muscle wasting and cardiac defects, as well as non-neuromuscular symptoms, such as hypogonadism, cataract, insulin resistance and premature male baldness.[80] There are two types of the disease (DM1 and DM2), for which the symptoms largely overlap. Both diseases are caused by nucleotide expansions.[81,82] In DM1, the expansion involves a CUG trinucleotide repeat located in the 3′ UTR of the DM protein kinase gene (*DMPK*).[82] DM2 involves a CCUG expansion in the first intron of the zinc finger 9 (*ZNF9*) gene.[81] The functions of the DM protein kinase and zinc finger protein 9 differ vastly, which is surprising given the overlapping symptoms. However, DM1 and DM2 are in all likelihood RNA-mediated diseases with a common disease pathology (Figure 4).[83] The expanded repeats in the mutant ZNF9 or DMPK transcripts form hairpin-like structures that are found in ribonuclear foci.[84] Here, they sequester a specific family of RNA-binding factors, the so-called muscleblind-like proteins (MBLN1-3).[85] This is accompanied by increased levels of another RNA-binding factor, the CUG-binding protein (CUGBP),[86] which could be attributed to CUGBP binding to soluble mutated RNAs (present outside foci).[87] This binding would increase the CUGBP half-life significantly, thus resulting in increased concentrations and activity. Decreased levels of MBLN and increased levels of CUGBP led to a synergistic misregulation of alternative splicing of a number of genes, including a muscle-specific chloride channel (*ClC-1*),[88] cardiac troponin 2 (*TNNT2*)[86] and the insulin receptor (*INSR*).[89] For each gene, the fetal isoform rather than the adult isoform is expressed in MD patients. There is strong evidence that the misregulation of certain transcripts leads to different aspects of the disease, for example, *ClC-1* misregulation leads to myotonia, *INSR* to insulin resistance and *TNNT2* to cardiac conduction defects.[83] Thus, restoring proper alternative splicing of one or more transcripts could have a therapeutic effect.

Antisense approaches for myotonic dystrophy

Misregulation of splicing of the *ClC-1* gene is (partially) responsible for one of the main features of DM (myotonia). In DM patients and mouse models, the alternatively spliced exon 7a is generally included (Figure 4). This exon disrupts the open reading frame leading to a premature stop codon in the constitutively included exon 7. Consequently, transcripts including exon 7a are subjected to NMD and do not result in ClC-1 protein. By contrast, functional protein can be translated from transcripts that do not contain exon 7a (as found in muscles from unaffected individuals). Wheeler *et al*[8] have used AONs targeting either the 3′ or 5′ splice sites of the aberrantly included exon 7a of the *ClC-1* gene in two mouse models. In the first model, ClC-1 splicing is disrupted because of a transgene with an expanded CUG repeat,[90] whereas in the other model this is the result of functionally knocking out MBNL1.[91,92] In both models, a single AON injection in the *tibialis anterior* muscle restored normal Cl–C1 splicing for at least 3 weeks, whereas lower levels of exon 7a skipping could be detected for over 8 weeks.[8] Exon 7a skipping was accompanied by increased ClC-1 protein levels at the sarcolemma, and current densities and deactivation kinetics as detected by patch clamp were restored to wild-type levels. EMG analysis showed a correlation between exon 7a skipping levels and the reduction of myotonia. Antisense-mediated exon 7a skipping may thus be a promising approach to treat one of the pathological hallmarks of DM. However, DM results from the misregulation of splicing of many genes and thus AONs targeting different genes would have to be combined to treat the different symptoms.

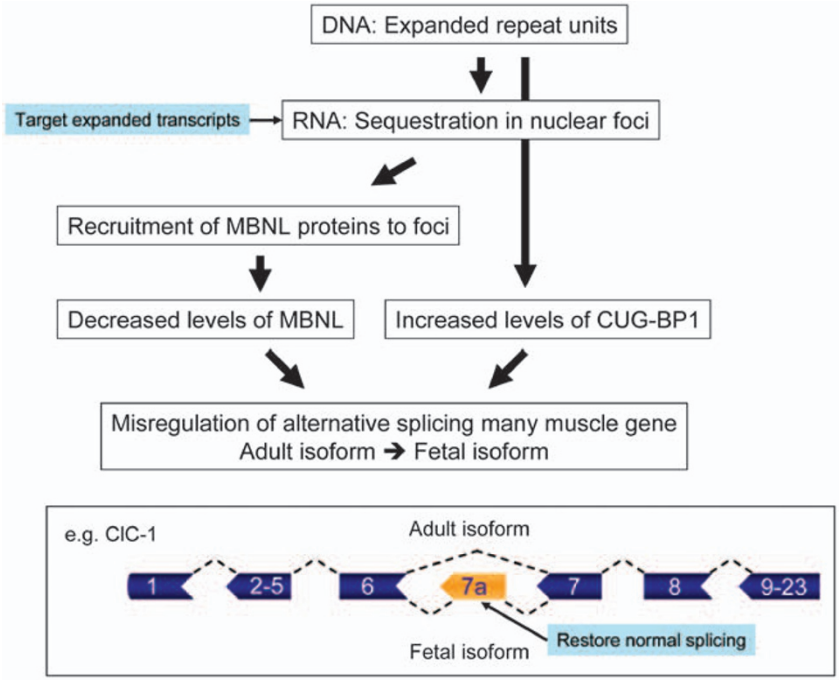

Figure 4 Overview of the pathology of myotonic dystrophy. Expanded repeat units eventually lead to a misregulation of alternative splicing (the CIC-1 gene is shown as an example). Therapeutic approaches can either target the expanded transcripts or try to restore normal splicing.

Alternatively, AONs are used to treat the cause of the misregulation, that is, the expanded repeats. Already in 1996, an attempt was made to inhibit *DMPK* expression in DM patient-derived cell cultures with DMPK-specific AONs that induced RNase H-mediated degradation.[93] However, this approach does not discriminate between wild-type and mutant transcripts and thus decreased overall DMPK levels, which by itself is considered to be detrimental.[83] A more specific result was reported by Krol *et al*[6] who treated DM patient-derived cells, interestingly in a relatively unspecific way using $(CAG)_7$ siRNA. Short CUG repeats are present throughout the human genome, and using CAG oligonucleotides might be expected to decrease expression of all $(CUG)_n$ containing transcripts. Surprisingly, this turned out not to be the case, as the wild-type DMPK transcript was unaffected, whereas only the level of the mutated transcript was significantly decreased.[6] The cause of this may be the relative molecular excess of the expanded repeat target (which can consist of hundreds or even thousands of CUGs) compared with the non-expanded allele and non-expanded other genes. In addition, the expanded repeats are thought to generate loops, which may increase the accessibility of the repeats compared with normal alleles. This approach could thus offer a therapeutic opportunity for other expanded repeat diseases as well. Recently, a 25mer CAG-repeat AON has been used to displace MBLN from the expanded repeats in foci in a DM mouse model.[94] This restored alternative splicing patterns of multiple genes, including ClC-1 and also reduced myotonia. A clinical trial to test this approach in DM patients is being planned (T Wheeler, personal communication).

CONCLUSION

This review provided an overview of how one tool can be tailored as a treatment for different disease mechanisms. The use of antisense oligonucleotides to modulate splicing has developed very fast during the last decade. Although recent experiments focused mainly on antisense-mediated exon skipping, it has now been shown that AONs can also be used to induce exon inclusion. In addition, the antisense approach can be used to induce specific knockdown of an expanded repeat. Proof-of-concept has been obtained for each of the different approaches and the current hurdle is delivery to the target tissues. In case of the dystrophies, AON uptake is facilitated by disease pathology, causing a damaged muscle membrane. However, for SMA, the AONs have to be delivered to the CNS, which may be more challenging, as the target is being protected by the blood- brain barrier. However, this may also be an advantage, as after intrathecal injection, the AON half-life in the CNS may be increased as they cannot exit the CNS.

It is noted that although for DMD the exon-skipping approach is mutation specific, for SMA and MD a single AON would be beneficial to all patients. Therefore, it is very likely that should the currently ongoing clinical trials with AONs for DMD prove successful, AONs will also be further developed for clinical applications for these NMDs.

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