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Splicing therapy for neuromuscular disease

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ABSTRACT

Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA) are two of the most common inherited neuromuscular diseases in humans. Both conditions are fatal and no clinically available treatments are able to significantly alter disease course in either case. However, by manipulation of pre-mRNA splicing using antisense oligonucleotides, defective transcripts from the *DMD* gene and from the *SMN2* gene in SMA can be modified to once again produce protein and restore function. A large number of *in vitro* and *in vivo* studies have validated the applicability of this approach and an increasing number of preliminary clinical trials have either been completed or are under way. Several different oligonucleotide chemistries can be used for this purpose and various strategies are being developed to facilitate increased delivery efficiency and prolonged therapeutic effect. As these novel therapeutic compounds start to enter the clinical arena, attention must also be drawn to the question of how best to facilitate the clinical development of such personalised genetic therapies and how best to implement their provision.

Introduction

Inherited neurological disorders have long suffered from a relative paucity of effective treatment options. Whilst our knowledge of supportive care along with symptomatic and palliative treatments has improved considerably over recent decades, the same has not been true of therapies aimed at the molecular defects themselves, despite many of the responsible genes and pathological mechanisms being known. This frustrating situation, however, is starting to change. We now know enough about the molecular pathogenesis of an increasing number of the monogenic neurological disorders to be able to design targeted disease-modifying genetic therapies for the first time. One such disease for which the development of targeted therapy is already far advanced is Duchenne muscular dystrophy (DMD). Another disease where such treatment is currently under rapid development is spinal muscular atrophy (SMA). This review will explain how the manipulation of RNA splicing can be used as an effective corrective therapy for these two classic genetic conditions. We outline the molecular pathogenesis and splicing biology of DMD and SMA, explain the design and use of antisense oligonucleotides in therapeutic exon skipping and exon inclusion respectively, and discuss the delivery of oligonucleotide drugs to muscle, heart and the central nervous system. We conclude with some thoughts on the future of splicing therapies in clinical practice.

Duchenne muscular dystrophy

DMD is a genetic disease of the muscle caused by mutations in the *DMD* gene, which lies at chromosomal locus Xp21 (Emery, 2002). The condition affects around 1 in 3500 live male births and generally presents in early childhood with proximal muscle weakness. Affected boys may present with gross motor delay and there can also be a non-progressive cognitive impairment of variable degree in around one third of cases. The usual natural history is one of gradually progressive weakness so that ambulation is lost by the teenage years. Histologically there is replacement of skeletal muscle tissue with fibrofatty infiltration (Zhou and Lu, 2010). This can result in a rubbery pseudohypertrophy of the calf muscles, which is a characteristic feature of the condition. The depleted muscle fibres show evidence of dystrophy, with repeating cycles of necrosis, regeneration and fibrosis resulting in unequal fibre size. The dystrophic process gradually affects the diaphragm and other respiratory muscles, eventually leading to respiratory failure, and cardiac muscle is also affected, resulting in a dilated cardiomyopathy (Fayssoil et al., 2010). Cardiorespiratory failure is the primary cause of mortality in such patients and death typically occurs in early adulthood. Current treatment options are limited, with supportive care and corticosteroid treatment being the mainstays of conventional therapy (Bushby et al., 2010a, 2010b; Moxley et al., 2010). Although advances in such

Abbreviations: 2′OMePS, 2′-O-methyl phosphorothioate; 2′MOE-PS, 2′-O-methoxyethyl phosphorothioate; AON, antisense oligonucleotide; CPP, cell-penetrating peptide; DMD, Duchenne muscular dystrophy; PMO, phosphorodiamidate morpholino; PPMO, peptide-conjugated phosphorodiamidate morpholino; SMA, spinal muscular atrophy.

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care have delivered considerable improvements in patient survival over recent decades, there remains a pressing need for disease-modifying therapy (Eagle et al., 2002).

Molecular pathogenesis of dystrophinopathies

The *DMD* gene encodes the protein dystrophin (Hoffman et al., 1987). At least seven major isoforms of differing lengths are encoded by this gene, each using an alternative intragenic promoter (Muntoni et al., 2003). The true number of isoforms is likely to be considerably higher, owing to the presence of multiple alternative splicing events. However, the full-length skeletal muscle isoform is a 427 kDa protein 3685 amino acids in length that localises to the sarcolemma (Zubrzycka-Gaarn et al., 1988). Here it plays a structural role, linking the cytoskeleton to the cell membrane and, *via* the dystrophin-associated glycoprotein complex (DAGC), beyond to the extracellular matrix. This connective function allows for the transmission of force from the contractile cytoskeletal elements of skeletal myofibres to extracellular structures. It is also important for maintaining the integrity of the muscle cell membrane (Davies and Nowak, 2006). The structure of full-length dystrophin allows it to carry out this role (see Fig. 1). On a simplistic level, the protein can be thought of as something akin to a bungee rope in that its central portion consists of a long, repetitive "rope-like" region (called the rod domain), whilst at either end there are molecular "hooks" to allow binding to cytoskeletal F-actin at one end (the N-terminus) and to the sarcolemmal DAGC at the other (the C-terminus). The rod domain is a coiled-coil region made of 24 spectrin-like repeats interspersed by 4 hinge regions (Ervasti, 2007). Although the rod domain is generally believed to have a large degree of functional redundancy in terms of dystrophin's mechanical role, it does contain a further actin-binding domain and is also thought to interact with membrane phospholipids, nNOS and other cytoskeletal elements such as plectin, intermediate filaments and microtubules (Le Rumeur et al., 2010). The major actin-binding interaction at the N-terminus is mediated by two calponin homology domains. At the other end of the protein, just proximal to the C-terminus, a strong interaction takes place with the β-dystroglycan component of the DAGC *via* a cysteine-rich domain. The C-terminus

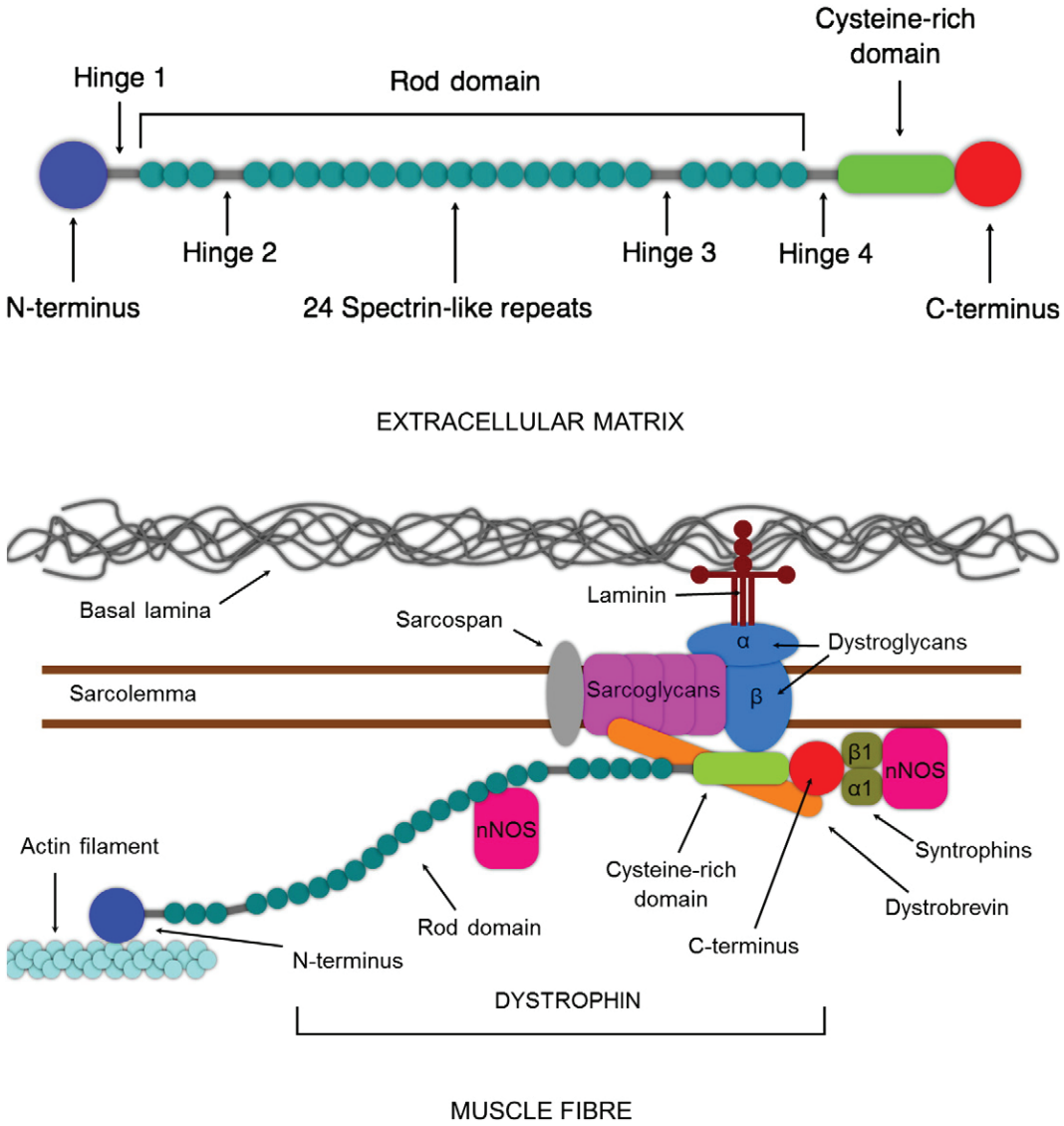

Fig. 1. Dystrophin and the dystrophin-associated glycoprotein complex (DAGC). Top: features of the dystrophin protein. The N- and C-terminal regions contain functionally important binding sites whilst the rod domain acts as a linker. The central rod domain comprises 24 spectrin-like repeats interspersed by 4 hinge regions that are thought to help provide molecular flexibility. In addition, the rod domain contains further binding sites for actin and nNOS. Bottom: dystrophin connects the cytoskeleton to the sarcolemma *via* components of the DAGC, a large multiprotein complex which includes laminin, sarcoglycans, α- and β-dystroglycan, sarcospan, dystrobrevin and α1- and β1-syntrophin, as well as associated proteins such as NOS. The N-terminus binds to F-actin, whilst at the other end the cysteine-rich region binds β-dystroglycan and the C-terminus binds to syntrophins and

itself binds other DAGC components such as syntrophins and α-dystrobrevin.

With this model in mind, it can easily be appreciated that a mutated dystrophin protein lacking either of its terminal ends would be functionally incapable. Indeed DMD patients usually have mutations that cause loss of the C-terminal domain, resulting in non-functional dystrophin. However, if a mutation were to lack only part of the central rod domain, the resulting protein could potentially still function tolerably well. This is borne out by the existence of the much milder condition Becker muscular dystrophy (BMD), which is allelic to DMD (Kingston et al., 1983). In BMD, patients typically express a truncated dystrophin protein that lacks a portion of the rod domain. Symptoms usually do not occur until late childhood, adolescence or adulthood and are generally a lot milder than DMD, with patients often maintaining ambulation well into middle and older age (Bushby and Garner-Medwin, 1993). As will be explained later, the presence of this milder phenotypic form of the disease provides the basis for how DMD can be treated by splice modulation.

The 2.4 Mb long *DMD* gene, located at cytogenetic locus Xp21.2–p21.1, has the distinction of being the longest known gene in the human genome (Boyce et al., 1991; den Dunnen et al., 1989). Seventy-nine exons are encoded by the full-length transcript (Roberts et al., 1993). However, despite its expansive genomic length, the fully spliced mature mRNA of full-length dystrophin is only some 14 kb long (Koenig et al., 1987). The implication of this is that whilst each exon is roughly of the order of 150 bp long (excluding the 2.3 kb final exon), they are separated by much longer introns that on average are about 30 kb long (although their individual lengths range from 107 bp for intron 14 up to over 319 kb for intron 1 of the brain full-length isoform). This feature of the *DMD* locus is likely to partly explain why single or multiple whole exon deletions are the most commonly found mutations in affected patients.

Since exon–exon junctions do not always fall neatly at the ends of triplet base codons, deletions of certain exons can cause a shift of the open reading frame at the site of the new exon–exon junction in the spliced transcript (see Fig. 2). Such frameshifts invariably lead to the incorporation of a premature STOP codon in the near downstream region of the transcript. If such a dystrophin protein were to be made, it would be nonfunctional and possibly unstable and thus these out-of-frame mutations cause DMD. In addition, the presence of a premature termination codon in the middle of a transcript is recognised by the cell, which activates the nonsense-mediated mRNA decay pathway, degrading the transcript and curtailing production of aberrant protein (Buvoli et al., 2007). In contrast to this, if exon deletions occur where the normal open reading frame is maintained, functional dystrophin is usually still produced, resulting in the milder BMD phenotype (Monaco et al., 1988).

Splicing biology of dystrophin

The extreme length of the *DMD* locus means that the RNA polymerase II enzyme takes around 16 hours to generate a single complete transcript (Tennyson et al., 1995). If a cell were to require the completion of the gene's transcription prior to splicing it, the whole process would be impossibly unwieldy. By necessity then, the cell commences pre-mRNA splicing concurrently with transcription. This co-transcriptional property appears to be a common feature of splicing in general and this impacts upon choice of available splice sites and on alternative exon selection (Kornblihtt et al., 2004). One interesting consequence of this prolonged time period needed for transcription is that rapidly dividing cells are unable to express dystrophin to any significant degree. The average time between cell divisions for cultured human myoblasts is in fact also around 16–17 h and so it may be that the paucity of dystrophin expression in such cells could in part be linked to this transcriptional time limitation (Blau et al., 1985; Nudel et al., 1988). Indeed, such a large gene is likely to have highly complex splicing and much remains

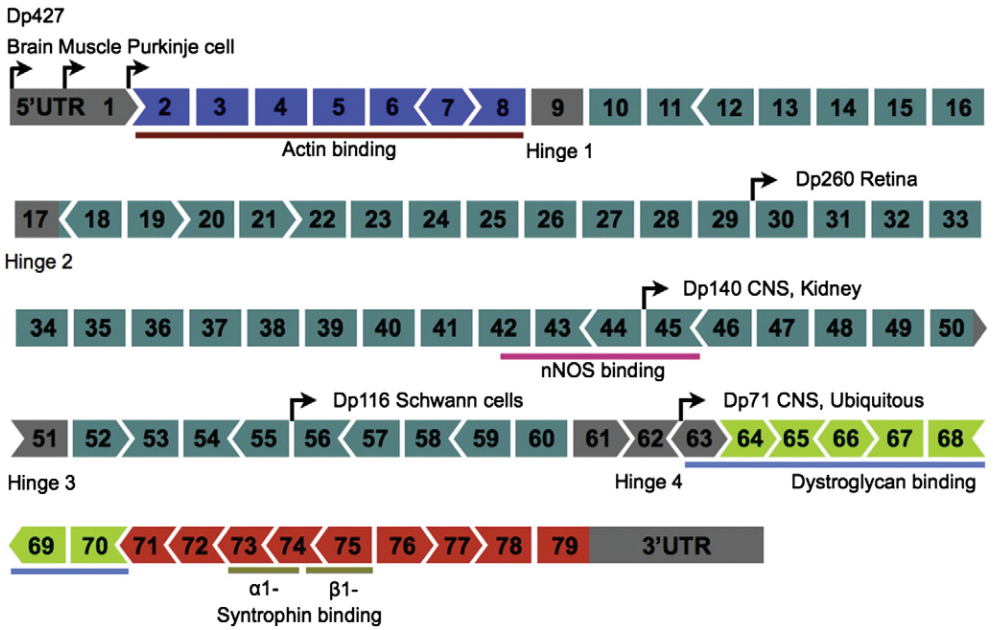

Fig. 2. Structure of the *DMD* gene, including positions of promoters for different isoforms. The full-length dystrophin protein (Dp427) has three separate tissue-specific promoters, predominantly expressed in brain (also known as the cortical promoter), skeletal muscle and cerebellar Purkinje cells respectively. The Dp260 isoform is mainly expressed in retina and originates from a promoter within intron 29. Dp140 has a promoter in intron 44 and is present in the CNS and kidney, whilst Dp116 has an intron 55 promoter and is predominantly found in Schwann cells. Dp71 is expressed from an intron 62 promoter and is ubiquitously expressed, although it appears to play an especially important role within the CNS. Also shown are the locations of binding sites for actin, nNOS, dystroglycans and syntrophins. The diagram also shows how the 79 exons fit together in terms of the normal open reading frame. Each individual exon may coincide with positions 1, 2 or 3 of a codon in the normal open reading frame. This is represented by three alternative shapes at the ends of the exons. If the exons fit together, the reading frame is maintained. If an exon (or a block of exons) with differently shaped ends is deleted from the gene, the reading frame is disrupted and the result is DMD. However, deletion of an exon with ends of the same configuration will not affect the reading frame since the remaining exons will still fit together (the equivalent of BMD). Exons are coloured according to the domain they encode: N-terminus (blue), rod domain (dark green with hinge regions in grey), cysteine-rich

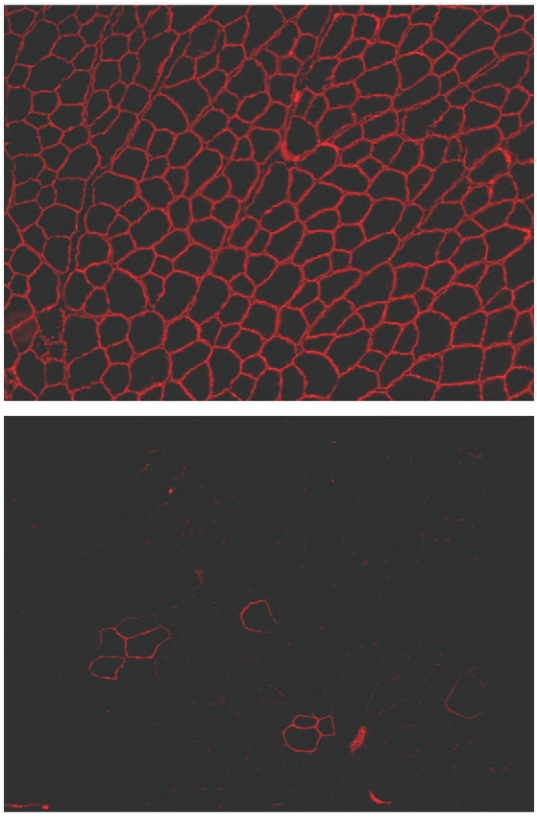

Fig. 3. Immunohistochemical fluorescent staining of dystrophin in skeletal muscle fibres (tibialis anterior) in mice. Top: sarcolemmal localisation of dystrophin in a wild-type (C57 BL/10) mouse. Transverse section of muscle fibres shows generally uniformly sized angulated fbres with consistent dystrophin staining around the cell membrane Bottom: lack of dystrophin expression in the *mdx* mouse DMD model. The majority of fibres show no dystrophin expression. However, note the presence of occasional revertant fibres in *mdx* leading to small clusters of dystrophin positive fibres, the result of sporadic naturally occurring exon-skipping events that restore the reading frame.

uncertain regarding the precise order of splicing events. It may be, for example, that shorter introns are spliced out more quickly than longer ones. This would lead to a nonconsecutive exon splicing order, which would have clear implications for splice-directed therapies (Aartsma-Rus et al., 2006). Some of the studies into DMD multi-exon splicing (discussed below) lend support to this hypothesis.

Immunohistochemical staining for dystrophin in the muscle tissue sections of DMD patients shows an absence of the protein from the muscle sarcolemmal membrane. However, occasional isolated fibres can be found that still appear to express correctly localised dystrophin (see Fig. 3) (Arechavala-Gomeza et al., 2010). These fibres, known as revertant fibres, are thought to be examples of where, by chance, second superadded mutations or intrinsic aberrant splicing events have led to the missing out or "skipping" of an additional exon or exons in a way that restores the original correct reading frame, allowing functional protein production (Lu et al., 2000; Klein et al., 1992).

Exon skipping in DMD

As can be seen from cases of BMD, loss of a substantial part of the dystrophin central rod domain can occur with relatively little impact on protein function. The idea behind splicing therapy in DMD is therefore to convert the out-of-frame transcript into an in-frame transcript that codes for functional protein. This can be achieved by the technique of inducing exon skipping in the mutant transcript, so as to bring it back into the original reading frame (Aartsma-Rus and van Ommen, 2007). Such exon skipping can be induced using antisense oligonucleotides (AONs). (generally not longer than 25 nt) and their sequences are designed so that they are complementary to a specific region on a pre-mRNA transcript of interest. The sequences usually target either a specific 5′ or 3′ splice site or else bind to a splicing regulatory element such as an intronic or exonic splicing enhancer (ISE or ESE) or intronic/exonic splicing silencer (ISS or ESS). Binding of an AON to the target sequence makes it unavailable to the spliceosome, interfering with the normal splicing mechanism. In this way it is possible to enhance either the inclusion or exclusion of a chosen exon from the mature mRNA (see Fig. 4). Some 70% of DMD mutations are intragenic exon deletions and are therefore potentially amenable to exon-skipping therapy (Aartsma-Rus et al., 2009a). Mutations are spread across the 79 *DMD* exons, however there are specific 'hotspot' regions where deletions are particularly common, such as between exons 45 and 55 where around 70% of deletions are located (Muntoni et al., 2003). AON sequences have in fact been designed for every internal DMD exon (Wilton et al., 2007). However, so far the majority of AON development has concentrated on skipping those individual exons that will benefit the greatest number of patients. Skipping exon 51, for example, can potentially be applied to 13% of all DMD mutations, exon 45 to 8.1% and exon 53 to 7.7% (Aartsma-Rus et al., 2009a). Exon skipping can also be used to treat nonsense mutations, which comprise around 15% of DMD mutations, by skipping the exon that contains the mutation itself (Spitali et al., 2009; Yokota et al., 2012). Of course, in such cases the reading frame must still be maintained and so single exon skipping for these mutations is limited to those exons that are not frame-shifting. However, this would still apply to around 47% of nonsense mutation patients.

Double and multi-exon skipping

Targeting single exons to skip can only ever hope to treat selected groups of DMD patients with amenable exon deletions. The exon reading frame structure of the gene means that some DMD mutations (including 47% of small point and frameshift mutations) require at least 2 exons to be skipped in order to restore or maintain the reading frame (*e.g.* an exon 8 deletion requires skipping of exons 6 and 7 and a point mutation in exon 69 or 70 requires that both these exons be skipped) (Aartsma-Rus et al., 2009a). Indeed single exon skipping as a technique can treat at best up to 64% of all DMD patients. However, if it is possible to skip 2 exons using 2 separate AONs (so-called double exon skipping), the proportion of treatable patients increases by 19%, meaning that a total of 83% of all DMD patients can be potentially treated by single or double exon skipping (Aartsma-Rus et al., 2009a).

If it were possible to effect the simultaneous skipping of more than 2 exons, a greater proportion of patients could potentially be treated using a repertoire of fewer therapeutic oligonucleotide compounds. This is because the skipping of a defined set of multiple exons can potentially correct the reading frame of multiple different exon deletions. At the same time it can also be used to treat point mutations in any of the skipped exons. For example, although the majority of DMD deletions occur between exons 45 and 55, it so happens that patients with specific deletions of this region in its entirety (exons 45–55 inclusive) are known to have particularly mild BMD phenotypes (Béroud et al., 2007). This makes it an ideal multi-exon skipping target. Recently it has been shown that bodywide restoration of dystrophin expression is in fact possible through multi-exon skipping of exons 45 to 55 using a cocktail of AONs (Aoki et al., 2012). This study was done in *mdx52* mice that lack *Dmd* exon 52 and utilised 10 separate AONs intravenously. In another study, double exon skipping of exons 43–44 was shown in cultured patient myotubes using separate AONs for each exon (Aartsma-Rus et al., 2004). Unexpectedly, skipping of the seven consecutive exons 45–51 was also achieved simply by using two AONs, one for exon 45 and the other for exon 51. The fact that this appears to work suggests that splicing of exons 45–50 may occur prior to exon 44–45 splicing. This is plausible

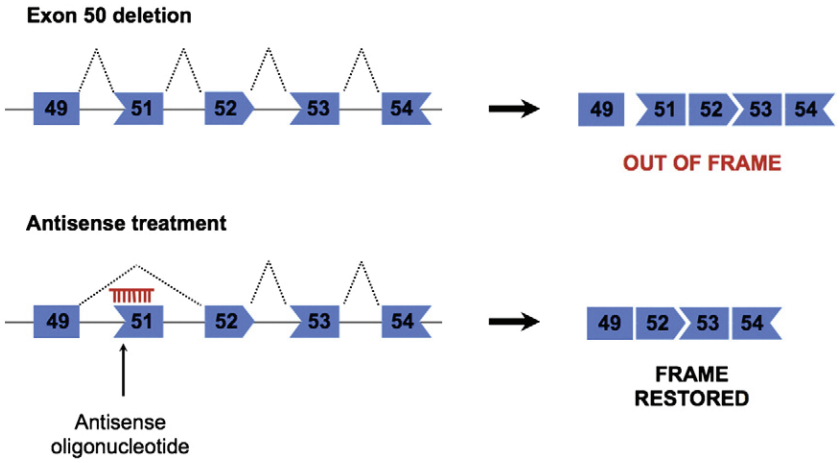

Fig. 4. The principle of antisense-induced exon skipping in DMD. In this example exon 50 of *DMD* is deleted, causing a frameshift in the resulting spliced mRNA. Addition of an AON that recognises and hybridises to a regulatory splicing element within the sequence of exon 51, such as an ESE, mediates skipping of this additional exon by the spliceosome. This corrects the reading frame in the spliced mRNA and restores dystrophin protein production. Although the resulting mRNA lacks an additional portion of the central rod domain, this has a minimal effect on overall protein function.

has been suggested for the finding that AONs targeting exon 8 always lead to double skipping of exons 8–9 (Aartsma-Rus and van Ommen, 2007). In this case intron 7 is 110 kb whilst intron 8 is only 1.1 kb.

Another use of multi-exon skipping is for the potential treatment of *DMD* exon duplications. Duplications, which make up around 5–15% of mutations (Muntoni et al., 2003), present a challenge to the AON approach since discriminating the extra copy from the original is generally not feasible and skipping both copies of the exon will often lead to a frameshift. However, by skipping an additional exon or exons, the reading frame can again be restored. An exon 44 duplication was amenable to induced exon 43–44 skipping using a combination of AONs in cultured muscle cells (Aartsma-Rus et al., 2007). However, the effects of multi-exon skipping on duplications are difficult to predict and depend on which exon is in question. For example, an exon 45 duplication only required a single AON targeting exon 45. However, a larger duplication of exons 52–62 proved refractory.

AON chemistry and design

In order to be effective therapeutic agents for the modulation of splicing, AONs ideally require a number of intrinsic properties. To start off with, the AON in question should bind in a sequence-specific manner to the target RNA transcript; the higher the specificity, the less the chance of unwanted off-target effects. Secondly, the AON should be of a chemistry that facilitates cellular uptake and activity in the appropriate intracellular compartment. Since splicing takes place in the nucleus, it is vital to design an AON that localises to the nucleus once it is taken up. In contrast, an antisense strategy seeking to utilise the siRNA pathway would best be served by an AON that remained in the cytoplasm where the processes of RNAi take place. Thirdly, because of the plethora of nucleases present *in vivo*, a well-designed AON should be resistant to nuclease degradation in order to allow it to reach its desired target intact and to maximise its potential duration of action once there. In addition to single-stranded stability, of particular importance for modified AONs is their interaction with RNAse H, which degrades RNA bound in RNA/DNA heteroduplexes. If the desired effect is transcript knock-down by degradation, the AON should be sensitive to RNAse H when bound to its target. However, for steric blocking techniques like exon skipping, the AON/RNA duplex should be resistant and not form a substrate for this enzyme. Fourthly, as with any drug, the ideal AON should have favourable pharmacokinetics and pharmacodynamics. Linked to this is of course the prerequisite that the AON should not effective delivery to the target tissues, whether that be a localised area such as a specific organ or brain region or body-wide systemic delivery such as to the musculature.

An ever-increasing range of different oligonucleotide chemistries have been developed to try to cope with these desired AON properties (Deleavey and Damha, 2012; Dias and Stein, 2002; Saleh et al., 2012). To date, several AON chemistries in particular have been utilised for splicing manipulation in DMD and SMA: 2′-O-methyl phosphorothioate (2′OMePS), 2′-O-methoxyethyl phosphorothioate (2′MOE-PS), phosphorodiamidate morpholino (PMO) and peptide nucleic acid (PNA) (see Fig. 5). Critically, in all these chemistries the ability to form Watson–Crick base-pairing with RNA is retained through the maintenance of the nitrogenous nucleobases in the correct spatial conformation. The backbone structures of these compounds, however, differ widely.

Phosphorothioates (which include 2′OMePS and 2′MOE-PS) are more closely related in structure to RNA than PMO or PNA. However, instead of utilising a phosphodiester link between nucleotides, the non-bridging oxygen atom of the phosphate group of RNA is substituted by a sulphur atom. This creates nuclease resistance and also generates chirality around the phosphorus atom, allowing formation of stereoisomers. Only the Sp diastereomer is, in fact, nuclease resistant, whilst the Rp diastereomer remains sensitive (Eckstein, 2002). Importantly, the phosphorothioate backbone modification does not, of itself, confer RNase H resistance. This is believed to be because the phosphorothioate/RNA heteroduplex adopts a conformation somewhere between B-form DNA and A-form dsRNA, which is therefore recognised by RNase H since its conformation approximates that of an RNA/DNA heteroduplex (Noy et al., 2008). Thus, resistance to RNase H instead requires 2′-O-modifications of the ribose residue, which tend to encourage a more dsRNA-like A-form conformation when bound in a heteroduplex with RNA (Deleavey and Damha, 2012). By adding a methoxyethyl group instead of a simple methyl group, 2′MOE-PS further increase nuclease resistance compared to 2′OMePS and also increase target RNA binding affinity, raising their melting temperature. Phosphorothioates also retain a negative charge. This greatly aids their solubility and means that they can be complexed easily together with cationic lipids and proteins. They also bind to plasma proteins in the circulation, which can significantly increase their half-life (Bennett and Swayze, 2010). However, they cannot be readily conjugated by covalent means to peptides. The mechanism by which they bind plasma proteins has yet to be fully elucidated but may be partly electrostatic or involve the formation

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