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Targeted Suppression of a Dystrophin Pseudo-exon using Antisense Oligonucleotides

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Abstract

Antisense oligonucleotide induced exon skipping is showing great promise as a potential therapy to restore functional dystrophin expression in Duchenne muscular dystrophy. Redirecting mRNA processing can excise one or more exons flanking frame-shifting deletions or duplications so that the reading frame is restored and a Becker muscular dystrophy-like transcript is induced. Here, we report the application of exon skipping to excise a disease-causing 72 bp pseudo-exon from the mature dystrophin mRNA. This pseudo-exon arises from a single T>G base change in intron 47 that creates an acceptor site with a strong consensus splice site score. Although in-frame, this pseudo-exon contains several stop codons and its inclusion is consistent with a diagnosis of Duchenne muscular dystrophy. The 21 year old patient is no longer ambulant but can stand and sit unassisted, indicating leaky pre-mRNA processing and the generation of some normal transcripts has delayed the muscle wasting to some extent. In cases where the DMD lesion activates one or more cryptic splice sites and results in the retention of intronic sequences in that mature mRNA, exon skipping has the potential to generate a normal gene transcript. Antisense oligonucleotides targeted to the mutant splice site were able to remove the pseudo-exon from the mature transcript and restore full-length dystrophin expression. This is in contrast to most exon-skipping strategies for Duchenne muscular dystrophy in which antisense oligonucleotides are used to restore expression but the induced Becker muscular dystrophy-like transcript is shorter and potentially less functional than the full-length mRNA. As such, pseudo-exons may be particularly amenable to exon skipping, especially when basal levels of expression have raised endogenous dystrophin levels, reducing disease severity and should mitigate immune responses to the induced normal isoform.

Keywords: Pseudo-exon; Exon skipping; Antisense oligonucleotide; Dystrophin gene; Phosphorodiamidate morpholino oligomer; Duchenne muscular dystrophy

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, severe muscle wasting disease caused by protein-truncating mutations in the dystrophin (DMD) gene. Becker muscular dystrophy (BMD) is an allelic but less severe disease, generally caused by deletions of one or more exons in the dystrophin gene that retain the open reading frame and allow production of semi-functional protein [1].

Antisense oligonucleotides (AO) with selected modified bases and backbone chemistries can be used as splice switching agents to excise specific exons, presumably through disruption or masking of motifs involved in the normal splicing of the dystrophin pre-mRNA. Targeted exon skipping can either restore the disrupted reading frame or excise in-frame exons carrying stop codons, and this strategy is emerging as a promising therapy for DMD [2,3]. Restoring the dystrophin reading frame in the most common type of dystrophin gene lesion, exonic deletions that disrupt the reading frame, allows a partially functional BMD-like dystrophin isoform to be translated from an otherwise defective gene disease.

Exclusion of exon 51 was chosen for the first DMD clinical trials, as this would restore the reading frame in about 13% of DMD exon deletion patients, and hence be relevant to most individuals [4]. Excising exon 51 in amenable patients should restore the reading frame and allow synthesis of BMD-like dystrophin isoforms. However, even if 100% efficient and administered to the appropriate DMD patient before onset of any pathology, a "cure" cannot be anticipated as the functionality of the BMD-like dystrophin is not normal and will depend upon the nature and extent of the primary DMD-causing gene lesion. Although induction of a BMD-like dystrophin has been shown to be clinically relevant in the Sarepta Therapeutics sponsored trial of eteplirsen, a phosphorodiamidate-morpholino oligomer designed to excise exon 51 [3], it is not a normal protein. Although it appears that the catastrophic decline associated with DMD has been slowed, the trial participants will have the milder condition of BMD, as long as the drug is administered and continues to show efficacy.

However, some rare DMD mutations offer the possibility of inducing a normal dystrophin gene transcript after targeted exon skipping. This study describes a deep intronic mutation that led to the retention of 72 bases of intron 47 in the mature dystrophin gene transcript. The mutation was reported previously [5], and although the patient was ambulant at 15 years of age, he was diagnosed with DMD because of the absence of dystrophin, as determined by Western blotting at that time. Although the pseudo-exon is in-frame, there are several in-frame stop codons that would result in premature termination of translation, consistent with a molecular diagnosis of DMD. Now aged 21, the patient is non-ambulatory but can sit and stand independently. It was deemed likely that low levels of functional

dystrophin are being expressed in this patient; despite the primary gene lesion and that this is due to imprecise or "leaky" splicing involving the recognition and retention of the pseudo-exon in the mature DMD transcript.

We recently described leaky expression involving DMD mutations in the 5' minor deletion hotspot [6], where exons 8 and 9 were frequently missing from the dystrophin transcripts from DMD/BMD patients diagnosed with frame-shifting deletions of exons 3-7 or 5-7. We demonstrated that exon 8 was particularly easy to excise from the mature dystrophin mRNA, and while targeting the acceptor splice site induced the most efficient exon skipping, every other AO evaluated induced some skipping [6]. We hypothesized that the leaky dystrophin expression involving exon 8 reflected the ease with which this exon could be removed with different AOs. We predicted that dystrophin pseudo-exons exhibiting similar leaky expression would also be simple to remove from the mature mRNA with AOs. We show that there are indeed low levels of natural pseudo-exon skipping in cultured cells from this patient, but contrary to expectations, this intron 47 pseudo-exon could only be efficiently removed with AOs targeted within a narrow region. Nevertheless, once optimized, the AOs did induce efficient exon skipping and we suggest that this type of mutation could indicate optimal standards for treating DMD by exon skipping. The low levels of dystrophin expression should eliminate the possibility of immune responses to the induced dystrophin, and the fully functional normal dystrophin could indicate minimum levels of induced protein necessary for clinical benefits.

Methods

AO design and synthesis

Oligomers consisting of 2′-O-methyl modified bases on a phosphorothioate backbone were designed to bind to the pseudo-exon and flanking sequences in intron 47, and synthesized on an Expedite 8909 synthesizer, as described by Adams et al. [7]. AO sequences and nomenclature are shown in Table 1, with 25mers used in the first pass and then microwalking undertaken to ascertain if further exon skipping potency can be achieved.

AO Number	AO coordinates	Sequence (5'-3)
1	Hi47A(-05+20)	caccuggcuccacccuugaccugug
2	Hi47A(+22+46)	auccucuuccaugauucaauuaucu
3	Hi47D(+11-14)	cuugugaaacuuacugauuaucagg
4	Hi47A(+05+29)	aauuaucugcaccuggcuccacccu
5	Hi47A(-01+24)	ucugcaccuggcuccacccuugacc
6	Hi47A(-10+15)	ggcuccacccuugaccugugggau
7	Hi47A(-15+10)	cacccuugaccugugggauuacca
8	Hi47A(-20+5)	uugaccugugggauuaccauaauu
9	Hi47A(-25-01)	cugugggauuaccauaauucaagg
10	Hi47A(+47+72)	ugauuaucaggagaacaguaugggg
11	Hi47A (10+20)	caccuggcuccacccuugaccugugggau
12	Hi47A(-5+25)	aucugcaccuggcuccacccuugaccugug
13	Hi47A(-01+29)	aauuaucugcaccuggcuccacccuugacc
14	Hi47A(-02+20)	caccuggcuccacccuugaccu
15	Hi47A(-05+17)	cuggcuccacccuugaccugug
16	Hi47A(+1+22)	ugcaccuggcuccacccuugac
17	Hi47A(+5+26)	uaucugcaccuggcuccacccu

Table 1: AO sequences used in this study and annealing coordinates relative to the pseudo-exon acceptor (A) and donor (D) splice sites; The AO nomenclature is based on that described previously [24] with the Hi47 referring to Human dystrophin intron 47 sequence and the numbers indicating intronic sequences either side of the pseudo-exon.

Cocktails of AOs were also evaluated, with details of the AO combinations shown in Table 2.

Phosphorodiamidate morpholino oligomers (PMO) were supplied by Sarepta Therapeutics, Cambridge, MA.

Cocktail number	AO-one	AO-two
1	1. Hi47A(-05+20)	2. Hi47A(+22+46)
2	1. Hi47A(-05+20)	10. Hi47A(+47+72)
3	2. Hi47A(+22+46)	6. Hi47A(-10+15)
4	2. Hi47A(+22+46)	8. Hi47A(-20+5)
5	2. Hi47A(+22+46)	9. Hi47A(-25-01)
6	3. Hi47D(+11-14)	4. Hi47A(+05+29)
7	3. Hi47D(+11-14)	6. Hi47A(-10+15)
8	3. Hi47D(+11-14)	8. Hi47A(-20+5)

Table 2: AO cocktails targeting DMD pseudo-exon in intron 47.

Cell Culture

Primary fibroblasts were derived from a skin biopsy taken from the DMD patient with a pseudo-exon mutation in intron 47 of the dystrophin gene, after informed consent. Prior to transfection, patient fibroblasts were induced into the myogenic lineage with a MyoD expressing adenovirus, Ad5.f50.AdApt.MyoD (Native Antigen Company, Oxford, UK). This was achieved using a viral multiplicity of infection of 200 as described [8], and cells were then differentiated in Dulbecco's modified Eagle medium (DMEM) (Life Technologies) supplemented with 5% horse serum (Gibco, Life Technologies) for 72 hours. Cells were seeded at $3x10^4$ per well in 24 well plates that had been sequentially pre-treated for 1 hour with 50 μg/ml poly D-Lysine (Sigma, Sydney, Australia) and 100 μg/ml Matrigel (BD Biosciences, Sydney, Australia) as described [9].

Transfection

Patient cells were transfected with either 2'-O-methyl AO-Lipofectamine 2000 (L2K) (Life Technologies) complexed at a 1:1 ratio in Opti-MEM (Life Technologies) according to the manufacturer's instructions, or a PMO annealed to a complementary DNA oligomer "leash" and complexed with L2K as described by [10], in Opti-MEM.

Cells transfected with 2'-O-methyl AOs were left for 48 hours and cells treated with the PMO: DNA lipoplexes were incubated for 96 hours.

RNA extraction and RT-PCR

Trizol (Life Technologies) was used to extract total RNA from cultured cells, according to the manufacturer's guidelines. RT-PCR primers were designed to amplify exons 43 to 51, and a second set of primers amplifying exons 45-48 was used in a nested PCR. Primer sequences for cDNA synthesis and amplification are shown in Table 3. RT-PCR was performed using 100 ng of total RNA using the Superscript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies) as described by Wilton et al. [11]. After 35 cycles, a 1 µL aliquot was removed and used as template in a nested PCR for 30 cycles using AmpliTaq Gold (Applied Biosystems, Melbourne, Australia). Exon skipping efficiencies were assessed by resolving the RT-PCR products on a 2% agarose gel in TAE buffer with a 100 bp DNA ladder (Life Technologies) for product size estimation and densitometry analysis. Densitometry was performed by capturing gel images on a Chemi-Smart 3000 system (Vilber Lourmat), and Bio1D-software was used for analysis (Scientific Software Group, Provo, UT).

Primer name	Sequence 5'-3'
43F	CAGGAAGCTCTCTCCCAGC
45F	ACAGATGCCAGTATTCTACAGG
48R	CTGAACGTCAAATGGTCCTTC
51R	GTCACCCACCATCACCCTCTG

Table 3: Primer sequences used for RT-PCR analysis.

Results

In silico analysis of splice motif scores

An online splice site score calculator was used to assess the consequences of the mutation [http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html]. The average score for a consensus acceptor splice site for this program is 7.9. When analysed as a splice site, the normal dystrophin intron 47 "acceptor" sequence scored -3.7, whereas the mutant (ie c6913-4037T>G) acceptor splice sequence resulted in a score of 7.2. The donor splice site used by this pseudo-exon scored 6.2, the average donor score is 8.1. A potential branch point was predicted 31 bases upstream of the pseudo-exon acceptor splice site.

2'O-methyl AO induced exon skipping

Three oligonucleotides consisting of 2'-O-methyl modified bases on a phosphorothioate backbone (2OMe AO) were designed to target the cryptic acceptor splice sites, an intra-exonic motif, and the donor site of the pseudo-exon. A cocktail containing the two AOs targeting the acceptor and donor sites was also evaluated in an attempt to further enhance exon skipping efficiency. Of these transfections, AO 1, Hi47A(-5+20) alone induced consistent and robust pseudo-exon skipping (Figure 1). The combination of AO 1 and 3, targeting both acceptor and donor splice sites, was ineffective, indicating that AO 3 compromised AO 1 activity. There is no substantial sequence complementarity between these AOs that could contribute to duplex formation and compromise activity.

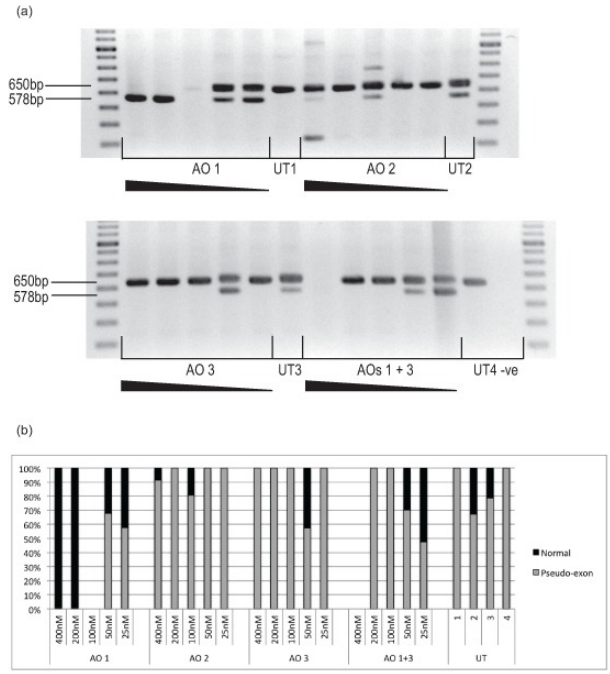
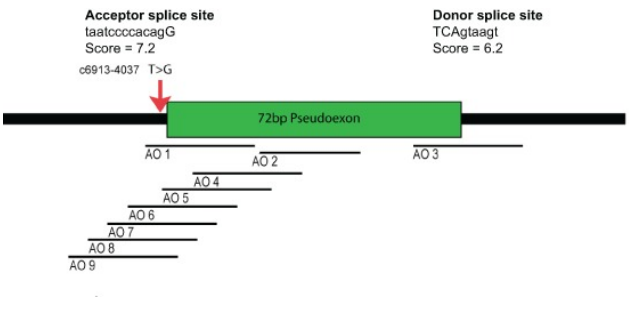


Figure 1: Nested RT-PCR analysis of dystrophin expression in DMD patient cells treated with pseudo-exon-targeting 2OMe AOs (n=2); (a) The patient's transcript amplicon containing the pseudo-exon is 650 bp in length, and skipping of the pseudo-exon is generates a 578 bp product. Each AO (and the AO cocktail) was tested at a total concentration of 400 nM, 200 nM, 100 nM, 50 nM and 25 nM (shown left to right). UT: untreated patient samples. AO 1: Hi47A (-05+20); AO2: Hi47A (+22+46); AO 3: Hi47D (+11-14); -ve: no template RT-PCR control. (b) Densitometry was used to analyse the relative amounts of mutant and normal transcripts in treated and untreated cells. Additional overlapping AOs were designed to anneal to the cryptic acceptor splice site, as shown in Figure 2.



Patient cells were transfected with AOs 4 to 9, prepared as cationic lipoplexes and incubated for 48 hours before RNA was extracted and the effects on splicing were assessed by RT-PCR. Of these AOs tested, only two, AO 4 (Hi47A (+05+29)) and AO 5 (Hi47A (-01+24)), induced consistent skipping of the pseudo-exon (Figure 3). The

remaining four AOs were generally ineffective and only induced sporadic skipping of the pseudo-exon, similar to that seen with AOs 2 and 3 in Figure 1 (data not shown).

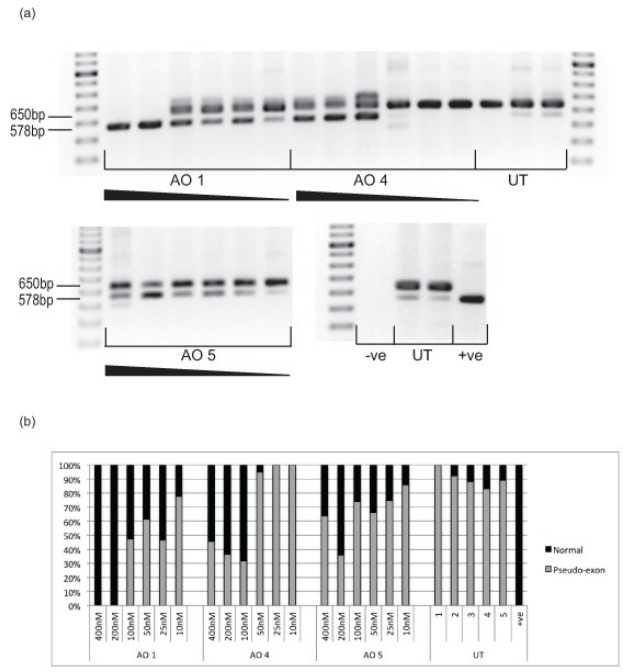


Figure 3: Nested RT-PCR analysis of dystrophin expression in DMD patient samples treated with AOs targeting the pseudo-exon. (a) The mutant transcript containing the pseudo-exon is 650 bp in length, and skipping of the pseudo-exon represents in a 578 bp amplicon. AOs were tested at 400 nM, 200 nM, 100 nM, 50 nM, 25 nM and 10 nM (shown left to right). UT: untreated patient samples. +ve: PCR amplification of normal dystrophin gene transcript. -ve: no template RT-PCR control. (b) Densitometry was used to analyse the relative amounts of mutant and normal transcripts in treated and untreated cells.

AO 1, Hi47A (-05+20), was the most efficient compound and induced of 100% skipping of the target exon at 400 nM and 200 nM (Figure 3). The overlapping AOs 4 and 5 also induced pseudo-exon skipping but were suboptimal when compared to AO 1. AO 4 induced robust exon skipping at the high concentrations (100-400 nM) but rapidly lost activity and with a pronounced threshold effect. AO 5 induced consistent pseudo-exon excision at all transfection concentrations, but complete suppression was not achieved, even at the higher concentrations.

AO cocktails consisting of pairs of non-overlapping AOs targeted to the pseudo-exon were evaluated (Table 2). All cocktails induced some pseudo-exon skipping, but none improved the levels above those achieved using AO 1 alone (data not shown). Eight new AOs, longer or shorter versions of the most efficient AO1, were evaluated, but none improved the result compared to AO 1 (data not shown).

PMO induced exon skipping

The most effective AO 1 sequence and an ineffective oligonucleotide (AO 6) were re-synthesized as PMOs to confirm extrapolation of exon skipping efficiencies were consistent with different splice switching oligomer chemistries (Figure 4).

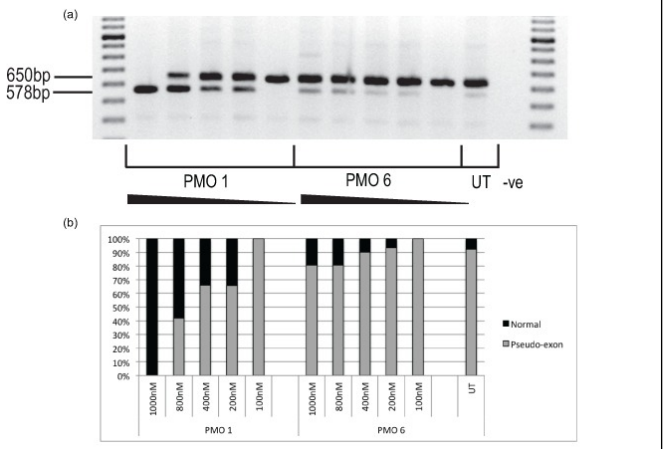


Figure 4: RT-PCR analysis of pseudo-exon 47 skipping in PMO-treated patient cells (n=2). (a) Each PMO was tested at 1000 nM, 800 nM, 400 nM, 200 nM and 100 nM (shown left to right). The full-length patient transcript product is 650bp in length, and skipping of the pseudo-exon generates a 578 bp amplicon. UT: untreated patient samples; -ve: no template RT-PCR control. (b) Densitometry was used to analyse the relative amount of mutant and normal transcripts in treated and untreated cells.

In silico analysis of splice switching AOs

The online tool SFOLD (http://sfold.wadsworth.org/cgi-bin/index.pl) was used to analyse target sequence folding and accessibility, before and after the addition of AOs (Supplementary Figure 1) This tool predicts mRNA secondary structure, with a score of 1.0 at a particular nucleotide predicting that base occupies a region in which the structure is open (does not interact with any other part of the mRNA), and 0 predicting that those bases are likely to be inaccessible to AOs due to interactions with another part of the RNA. Once an AO binding site is specified, that site appears with a score of 1.0, indicating it cannot bind to another region of the mRNA. The accessibility profile remains the same between normal sequence and that after AO 6 annealing, except at the region of AO binding. When the analysis was performed with AO 1, a predicted peak (indicated by a red asterix) is significantly reduced compared to normal (Figure 5). This smaller peak indicates a reduction in accessibility of this region and interrogation of the sequence with the two other AOs that induced some consistent pseudo-exon skipping (AOs 4 and 5) also saw a reduction in this peak, albeit less pronounced than AO 1. AOs 3, 6, 7 and 9 had no effect on the splicing pattern, and also did not affect the peak of interest.

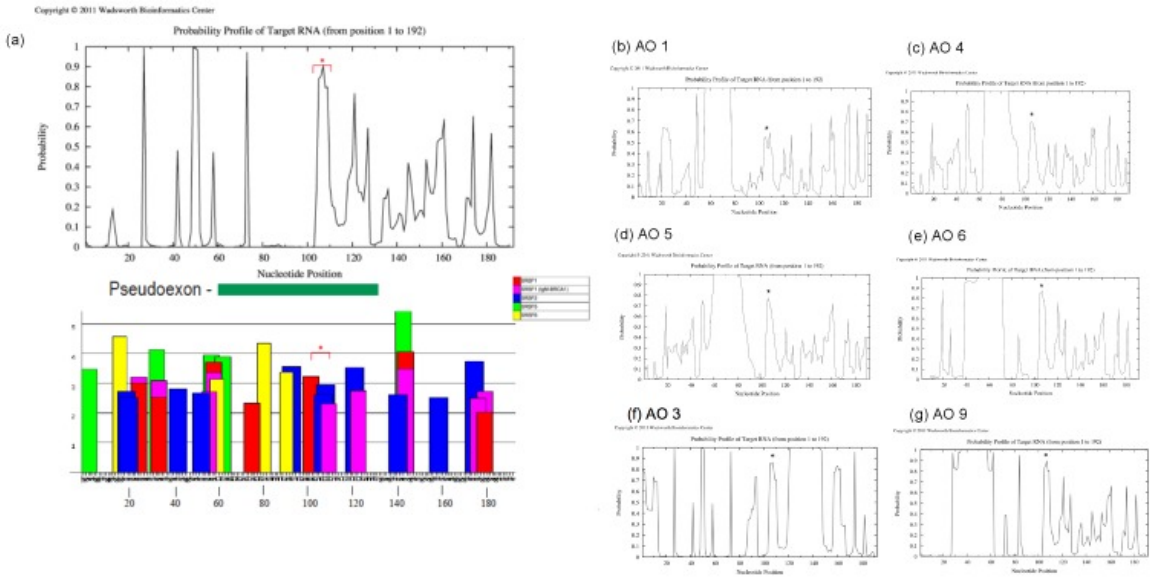


Figure 5: SFOLD analysis of the intronic region containing the pseudo-exon. The 72 bp pseudo-exon and 60 bp of intronic sequence on either side were used for analysis. The accessibility profile for normal transcripts is shown in (a), aligned with ESE sites predicted using ESE finder (http://rulai.cshl.edu/cgibin/tools/ESE3/esefinder.cgi?process=home). (b)- (g) show the predicted accessibility profile if AOs are bound to the transcript. The asterix indicates a region of interest.

Discussion

Protein truncating mutations in the dystrophin gene typically lead to DMD, but despite catastrophic mutations (frame-shifting deletions/duplications of one or more exons, intra-exonic insertions/deletions that disrupt the reading frame or nonsense mutations), low levels of dystrophin have been detected in two out of three DMD patients [12-14]. Although such basal expression of dystrophin presumably arises through natural exon skipping caused by imprecise dystrophin pre-mRNA processing, the levels are insufficient to prevent disease pathology or alter the clinical outcome.

Mutations in DMD that activate pseudo-exon retention in the mature dystrophin mRNA have been reported in introns 1, 9, 11, 44, 45, 47, 56, 60, 62 and 25 [5,15-17] as the result of point mutations activating cryptic donor and/or acceptor sites. In addition to point mutations, it has been established that the position of splice motifs in relation to one another is an important factor in exon recognition and splicing [18], so pseudo-exons can also arise if deletions bring previously distant splice motifs closer together.

In the majority of cases, individuals carrying dystrophin pseudo-exon activating mutations present with intermediate/BMD phenotypes, which is frequently inconsistent with a "molecular DNA diagnosis" of DMD. This indicates some laxity in the recognition and processing of the pseudo-exons, thereby allowing normal transcripts and protein to be generated. We recently reported fold increases in dystrophin expression in treated DMD patient cells carrying 'leaky' mutations in the minor DMD deletion hotspot [5]. Deletions of dystrophin exons 3-7 disrupt the reading frame and, while consistent with a molecular diagnosis of DMD, many individuals with this mutation present with an intermediate/BMD phenotype [12]. Similar to transcripts with pseudo-exon, transcripts from DMD alleles with a genomic deletion of exons 3-7 also frequently exclude exons 2 or 8 and 9 [19].

Gurvich et al. [5] first reported the intron 47 pseudo-exon mutation as well as two other out-of-frame pseudo-exon mutations, both caused by a single base change inducing cryptic donor splice sites; one in intron 11 (mild BMD phenotype), and the other in intron 45 (DMD) [5]. The patient with the intron 11 mutation and mild phenotype was found to express normal full-length dystrophin at 13% of normal levels [5]. Two AOs were designed for each pseudo-exon, one targeting the cryptic donor splice site and the other a predicted cluster of ESEs within each pseudo-exon, and all were able to excise the target exon from the mature transcripts. However, 100% exon skipping was only achieved when both AOs were combined and transfected into cells derived from the patient with the mutation in intron 11. This was in contrast to our observations in this study, where AO combination targeting pseudo-exon 47 was ineffective, or in the case of cocktails combining AO 1, counterproductive. We have observed pronounced synergy between some non-overlapping AO combinations [7], but this is the first time we have seen one AO compromising the efficiency of another. We previously hypothesized that the synergistic effect of some combinations arises after one AO anneals to the pre-mRNA and alters secondary or tertiary structures to facilitate binding of the other AO [20]. Perhaps a similar mechanism is in action in this case, except that annealing of AO 3 alters pre-mRNA structure such that AO 1 can no longer access the target binding site. Only 4 bases are complementary between these oligomers so it is unlikely that duplex pairing between the 2 compounds could contribute to the loss of AO 1 induced exon skipping. Some other AO cocktails induced pseudo-exon skipping with variable efficiency, but only up to 50% skipping at the higher concentrations (data not shown).

We have demonstrated that one PMO, synthesized with the same sequence as the most effective 2'O-methyl AO, excludes the intron 47

pseudo-exon from the transcript in a consistent, dose-dependent manner. Surprisingly, the PMO whose sequence was based on an ineffective 2'-O-methyl AO, was able to induce low levels of exon skipping in a dose-dependent manner, although the degree of exon skipping was not greatly elevated above the basal levels frequently observed in untreated cells. Although the annealing sites of the two PMOs overlapped, the differences in exon skipping efficiencies were pronounced and further support the finding of only a narrow window available for effective AO modification of splicing in this case. This is also consistent with our observations that the PMO chemistry is superior to the 2'-O-methyl AOs with respect to splice switching both *in vitro and in vivo*. Recently, we demonstrated that consistent multi-exon skipping, to correct the reading frame around DMD exon duplication mutations, could only be induced in a dose-dependent manner when using PMOs [21].

We demonstrate the need for careful AO design, particularly when the amenable target motifs for splice switching are relatively small. Our experience, at least with inducing dystrophin exon skipping is that the donor splice site rarely offers a good target [11]. In this case, the very first AO designed for skipping was the most efficient, and subsequent AO microwalking only resulted in compounds with reduced splice switching potential. High density screening is essential to ensure the most effective compound is identified for pre-clinical testing, and it is rare the most efficient AO is designed in the first pass. All of the canonical DMD exons can be removed from the mature transcript, but there is a large variation in efficiency; some exons are readily removed at low AO concentration, while others are not efficiently excluded, even at high concentrations [11]. The process of RNA splicing is complex and involves multiple *cis* factors, as well as binding proteins, and RNA folding during splicing is likely to contribute to the outcome [22,23]. It is now very evident that using AOs to redirect splicing is not as simple as blocking a crucial acceptor or donor splice site. It is also apparent that Tm of the AO alone, does not have a major impact on AO splice switching ability, and that the AO length and annealing site are important considerations in AO design [7].

In silico tools were used to assess splicing motifs and mRNA folding in the region of the pseudo-exon. Supplementary Figure 1 shows predicted splice motifs and predicted free energy of RNA structures using a sliding window. The analysis of the pseudo-exon and the surrounding region confirmed the predicted acceptor splice site and also indicates the donor site used. Two prediction tools were used, ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and Human Splice Finder (http://www.umd.be/HSF/). The analysis was run using the default parameters for both programs, but very different results were obtained. ESEfinder failed to predict the donor site utilised by this pseudo-exon, highlighting the limitations of these programs and indicating excessively stringent prediction parameters.

The peak of interest identified by the SFOLD analysis coincides with a predicted cluster of exon splicing enhancers, SRSF1 and 2, serine arginine rich splicing factors implicated in exon definition. It appears that the most efficient AO capable of inducing exon skipping reduces the accessibility of this region, thereby limiting splice factor binding and thus reducing exon recognition and retention in the mature mRNA. Less effective AOs do not reduce accessibility to the same extent, while completely ineffective AOs such as 2 and 10 do not reduce accessibility. It was of interest that 2 AO cocktails (AOs 1+3 and 1+10) failed to skip as efficiently as AO 1 alone. It is possible that AOs 3 and 10 are stabilizing some parts of the RNA structure so AO 1 cannot reduce accessibility of that peak and thereby compromise exon recognition.

In conclusion, we were able to demonstrate efficient removal of the pseudo-exon in intron 47 of the dystrophin gene in patient cells, although AO induced skipping was only achieved upon targeting a narrow mRNA region. The presence of a low level of natural exon skipping was evident following RT-PCR amplification of RNA from untreated cells, and the patient's phenotype reflects this data, suggesting a diagnosis of BMD is more appropriate than DMD. Although patient numbers are obviously limited, pseudo-exon mutations should be considered as ideal candidates for exon skipping clinical trials due to the efficiency of removal, the probability of low level basal expression and potential to generate a normal full-length dystrophin.

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