Dystrophin Isoform Induction *In Vivo* by Antisense-mediated Alternative Splicing

Sue Fletcher[1], Abbie M Adams[1], Russell D Johnsen[1], Kane Greer[1], Hong M Moulton[2] and Steve D Wilton[1]

[1]Centre for Neuromuscular and Neurological Disorders, University of Western Australia, Perth, Western Australia, Australia;
[2]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, USA

Antisense oligomer-induced manipulation of dystrophin pre-mRNA processing can remove exons carrying mutations, or exclude exons flanking frameshifting mutations, and restore dystrophin expression in dystrophinopathy models and in Duchenne muscular dystrophy (DMD) patients. Splice intervention can also be used to manipulate the normal dystrophin pre-mRNA processing and ablate dystrophin expression in wild-type mice, with signs of pathology being induced in selected muscles within 4 weeks of commencing treatment. The disruption of normal dystrophin pre-mRNA processing to alter the reading frame can be very efficient and offers an alternative mechanism to RNA silencing for gene suppression. In addition, it is possible to remove in-frame exon blocks from the *DMD* gene transcript and induce specific dystrophin isoforms that retain partial functionality, without having to generate transgenic animal models. Specific exon removal to yield in-frame dystrophin transcripts will facilitate mapping of functional protein domains, based upon exon boundaries, and will be particularly relevant where there is either limited, or conflicting information as to the consequences of in-frame dystrophin exon deletions on the clinical severity and progression of the dystrophinopathy.

INTRODUCTION

Elucidation of many of the molecular mechanisms that are important in disease provides opportunities for new bio-therapeutics. Antisense agents have proved to be valuable tools to inhibit gene expression by a variety of mechanisms, and to manipulate exon selection during primary transcript processing to overcome disease-causing gene lesions. We have developed antisense strategies to bypass disease-causing mutations in the *DMD* gene that lead to Duchenne muscular dystrophy (DMD), a relentlessly progressive muscle wasting disorder with a predictable course and fatal outcome (for review see ref. 1).

Although predominantly expressed at low levels, dystrophin serves a crucial structural role in muscle. Mutations that prevent synthesis of a functional dystrophin result in muscle fibers prone to mechanical damage, with secondary changes in signaling and metabolism, causing the severe muscle wasting and pathology associated with DMD (for review see ref. 2). Selected exon exclusion mediated by antisense oligomers can bypass premature termination codons[3–5] or restore the reading frame around frameshifting mutations in the *DMD* gene,[6–9] and can lead to functional protein expression and localization. Although whole-exon dystrophin deletions are clustered in hotspots,[10] nondeletion mutations are spread across the entire gene.[11] In-frame dystrophin deletions, particularly those in the central rod domain, generally result in the production of partially functional protein and the less severe, allelic disorder, Becker muscular dystrophy (BMD).[12] Examination of the genomic organization in mildly affected BMD patients provides templates for a number of potentially functional dystrophin isoforms. However, in-frame deletions in the latter third of the gene are rare[11] (**Figure 1**) and the optimal exon-skipping strategies to bypass many *DMD* gene mutations, and the functionality of the induced dystrophin isoforms[13] remain to be determined. The paucity of BMD-causing mutations in the 3′ region of the *DMD* gene probably reflects a combination of this area not being deletion-prone and the exon arrangement being such that most exon junctions occur within codons, thereby predisposing exon deletions or duplications to disrupting the reading frame.

Exclusion of an in-frame, multiexon block that maintains an open reading frame can result in a BMD-like protein,[9,14–19] and will allow functional mapping of dystrophin domains according to exon boundaries. This will assist in devising the optimal exon-skipping strategies to overcome DMD-causing mutations, particularly in those regions of the *DMD* gene that are not well represented in the dystrophin mutation databases. We now extend the application of selected exon removal to induce transient *in vivo* models to allow specific dystrophin isoforms to be evaluated. In addition, exclusion of a frameshifting exon is another mechanism for efficient inhibition of gene expression and may be a useful technique to study the consequences of gene knockdown *in vivo*.

RESULTS

Dystrophin exon arrangement

The dystrophin exon arrangement is shown in **Figure 1**, and the reading frame and encoded functional protein domains are indicated. The primary actin-binding domain 1 occurs within the first 90 amino acids.[20] An intact actin-binding domain 1 is required for normal muscle function and to protect myofibers from necrosis.[21]
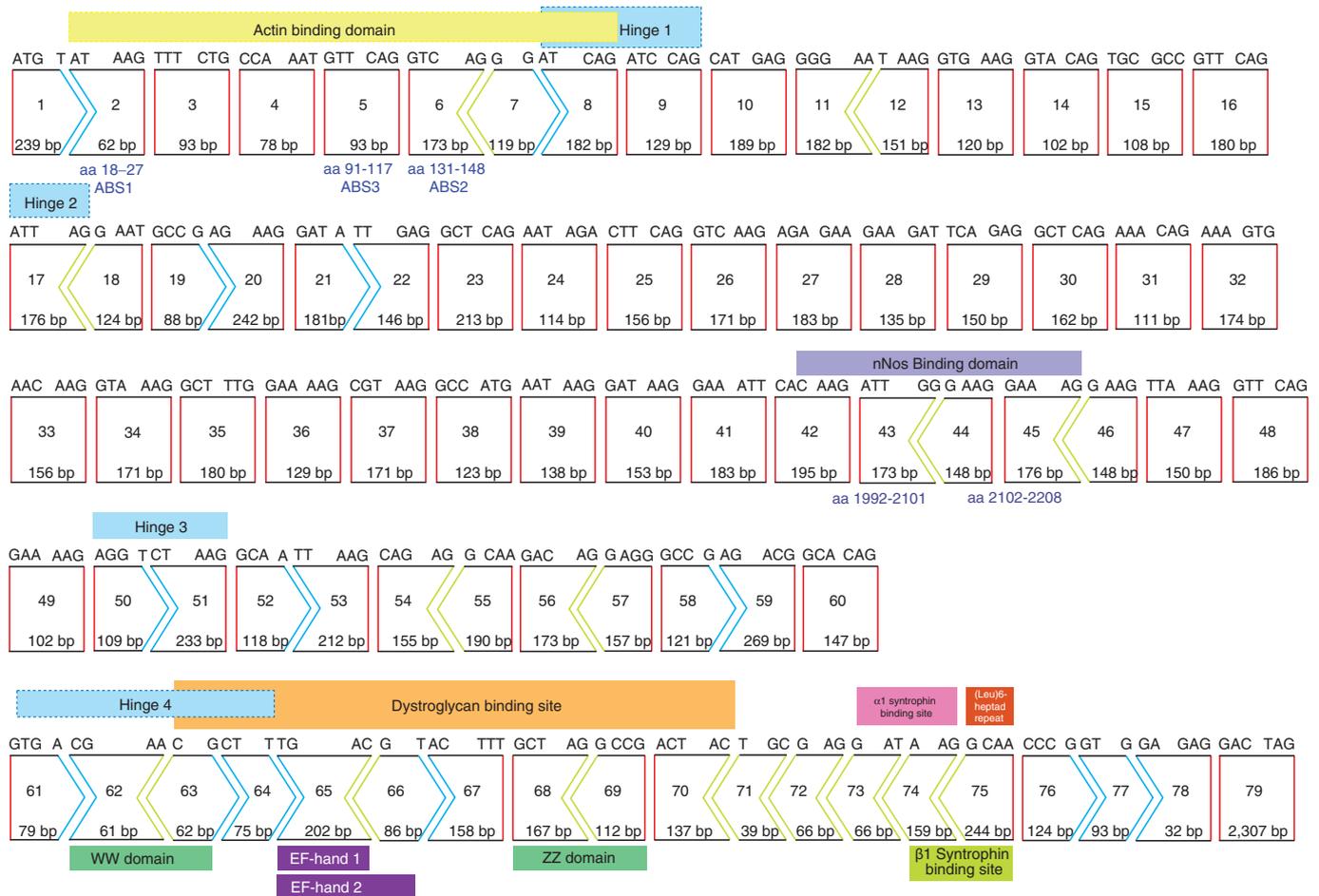
Figure 1 Dystrophin transcript structure,[43] showing the reading frame and major functional protein coding domains (actin-binding domain; exons 2–8,[20,21,44,45] central rod domain; exons 8–61, which includes nNos binding sites (exons 42–45),[46] cysteine-rich domain[22] (dystroglycan-binding site; EF1 domain and ZZ domain[23]) exons 63–70, the α-syntrophin binding site[24] and carboxy-terminal domain (exons 70–79). In-frame exons are indicated by rectangles (red sides), whereas codons disrupted by exon junctions are indicated by arrows.

Genomic deletions extending downstream of exon 55 are rare,[11] and it is apparent (**Figure 1**) that most deletions in this region will disrupt the reading frame and/or impact upon important functional domains. Deletion analysis indicates that the broad cysteine-rich domain is indispensable for dystrophin function and coincides with the *in vitro* identified β-dystroglycan-binding domain[22] found to include EF1 and ZZ domains[23] and the α-syntrophin binding site, coded for by exons 63–70,[24] while the carboxy terminal is not essential for assembly of the dystrophin-associated glycoprotein complex.[25] Thirty-nine of the exons are in-frame, and may be deleted individually without disrupting the reading frame.

Induction of a dystrophin isoform missing exons 19 and 20

Neonatal C57BL/10ScSn mice, 3–4 days old (*n* = 5) were injected twice-weekly, with peptide-conjugated phosphorodiamidate morpholino oligomers (PMOs) (20 mg/kg) targeting exon 19, and exons 19 and 20 simultaneously, via the intraperitoneal route. The animals were euthanized at 10 weeks of age and dystrophin expression in selected tissues was analyzed by reverse transcription (RT)-PCR, western blotting, and immunofluorescence.

The removal of exon 19 ablated dystrophin expression in diaphragm from treated C57BL/10ScSn mice, whereas the removal of exons 19 and 20 together render the diaphragm indistinguishable from wild-type, sham-treated mouse diaphragm (**Figure 2a**). RT-PCR analysis of dystrophin transcripts in tibialis anterior, diaphragm and heart from mice treated with oligomers targeting exon 19 and exons 19 and 20 together showed efficient splice-switching and generation of induced shortened transcripts in striated muscle, but not in heart (**Figure 2b**).

Induction of a dystrophin isoform missing exons 52 and 53

Neonatal C57BL/10ScSn mice, 2–4 days old (*n* = 5) were injected intraperitoneal twice-weekly, with peptide-conjugated PMOs (20 mg/kg) targeting exon 52, exon 53, and exons 52 and 53 simultaneously. Four weeks after commencing application of the oligomer targeting exon 53, severe dystrophic pathology with central nucleation, mononuclear infiltrate, and fibrosis was evident in the diaphragm. Efficient exclusion of exon 53 from the transcript was demonstrated by RT-PCR on RNA prepared from diaphragm, and to a lesser degree in tibialis anterior, but not in heart (**Figure 3b**) and the absence of dystrophin in diaphragm
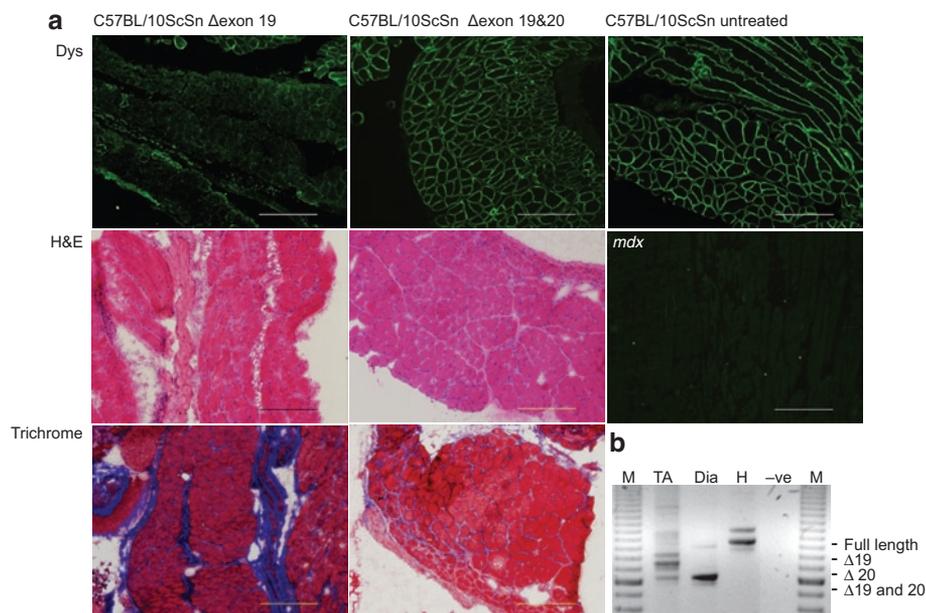
Figure 2 Analysis of dystrophin expression in selected tissues from 10-week-old C57BL/10ScSn mice injected i.p. twice-weekly with peptide-conjugated PMOs (20 mg/kg) targeting dystrophin exons 19 and 20. (a) Immunofluorescent detection of dystrophin on diaphragm cryosections from mice treated with oligomers targeting exon 19 (Δ19) and exons 19 and 20 (Δ19 and 20) (upper panel), haematoxylin and eosin staining (H&E) (middle panel), and Picro Mallory trichrome (lower panel) staining revealing muscle architecture. Sections from sham-treated C57BL/10ScSn and *mdx* mice are included for comparison (bar = 20 μm). **(b)** Nested RT-PCR analysis of dystrophin transcripts in tibialis anterior (TA), diaphragm (Dia), and heart (H) from mice treated with oligomers targeting exons 19 and 20 (Δ19 and 20). Transcript product sizes in base pairs (bp) are indicated (M = 100 bp marker). i.p., intraperitoneal; PMO, phosphorodiamidate morpholino oligomer; RT, reverse transcription.

was confirmed by western blotting (**Figure 3c**). Dystrophin exon 52 removal was less efficient, as shown by the residual dystrophin in **Figure 3c** and partial dystrophin staining of some fibers in **Figure 3a**. However, the simultaneous removal of exons 52 and 53 maintained the dystrophin reading frame (**Figure 1**) and normal sarcolemmal dystrophin localization and muscle architecture (**Figure 3a**). RT-PCR showed the absence of a product representing the full-length transcript after dual exon skipping, consistent with the western blot that shows near-normal levels of dystrophin in diaphragm after removal of exons 52 and 53. Diaphragm muscle architecture and dystrophin expression was almost indistinguishable from that of an age-matched, untreated wild-type mouse.

DISCUSSION

Intervention by splice switching is dependant upon several parameters including appropriate oligomer design and chemistry, and efficient oligomer uptake. The use of PMOs, which have demonstrated efficient and prolonged splice-switching action *in vivo*,[26,27] conjugated to cell-penetrating peptides for efficient systemic delivery[4,28–30] allows exploitation of AO-mediated exon selection to study protein function in animal models. We are generating in-frame transcripts to allow mapping of functional protein domains, based upon exon boundaries. Exclusion of an in-frame, multiexon block to maintain the reading frame permits functional assessment of the dystrophin isoforms and will assist in devising exon-skipping strategies for various DMD-causing mutations.

The genotype–phenotype data available in existing dystrophinopathy databases can be compromised by inaccurate mutation and clinical information and incomplete mutation type representation. Some of the apparent discrepancies to the reading-frame rule[12] in the databases are due to the limitations of diagnostic procedures available at the time of testing. Mutation confirmation at the RNA level reveals over 99.5% concordance with the reading-frame rule.[31] Completion of the TREAT-NMD database[32,33] will provide accurate information on defined mutations, permitting correlation of the genotype and clinical diagnosis, and assist in determining the best exon-skipping strategies for many dystrophin mutations. However, the paucity of BMD-causing mutations in some parts of the *DMD* gene, and in particular, the latter third, does not facilitate the design of exon-skipping strategies to bypass DMD-causing mutations in this region.

The first report of exon skipping in the dystrophin transcript described induction of abnormal splicing and exclusion of exon 19 in a patient with an intraexonic deletion.[34] Subsequently, an oligodeoxynucleotide targeted to exon 19 was administered to a patient with a deletion of exon 20 in an effort to restore the dystrophin reading frame.[35] There are only three records of deletions of dystrophin exons 19 and 20 in the Leiden database (www.dmd.nl/) and all are described as DMD. However, one diagnosis was carried by Southern blotting, another by multiplex PCR and follow-up investigations of the third found no record of the submitted mutation (K. Flanigan, personal communication).

The potential phenotype associated with the loss of exons 19 and 20 is not known, but would be predicted to be mild, because these exons occur within the rod domain region. Data presented here confirms that the dystrophin isoform translated from a transcript missing exons 19 and 20 is correctly localized to the sarcolemma and that the muscle architecture in diaphragm and tibialis anterior appears normal.
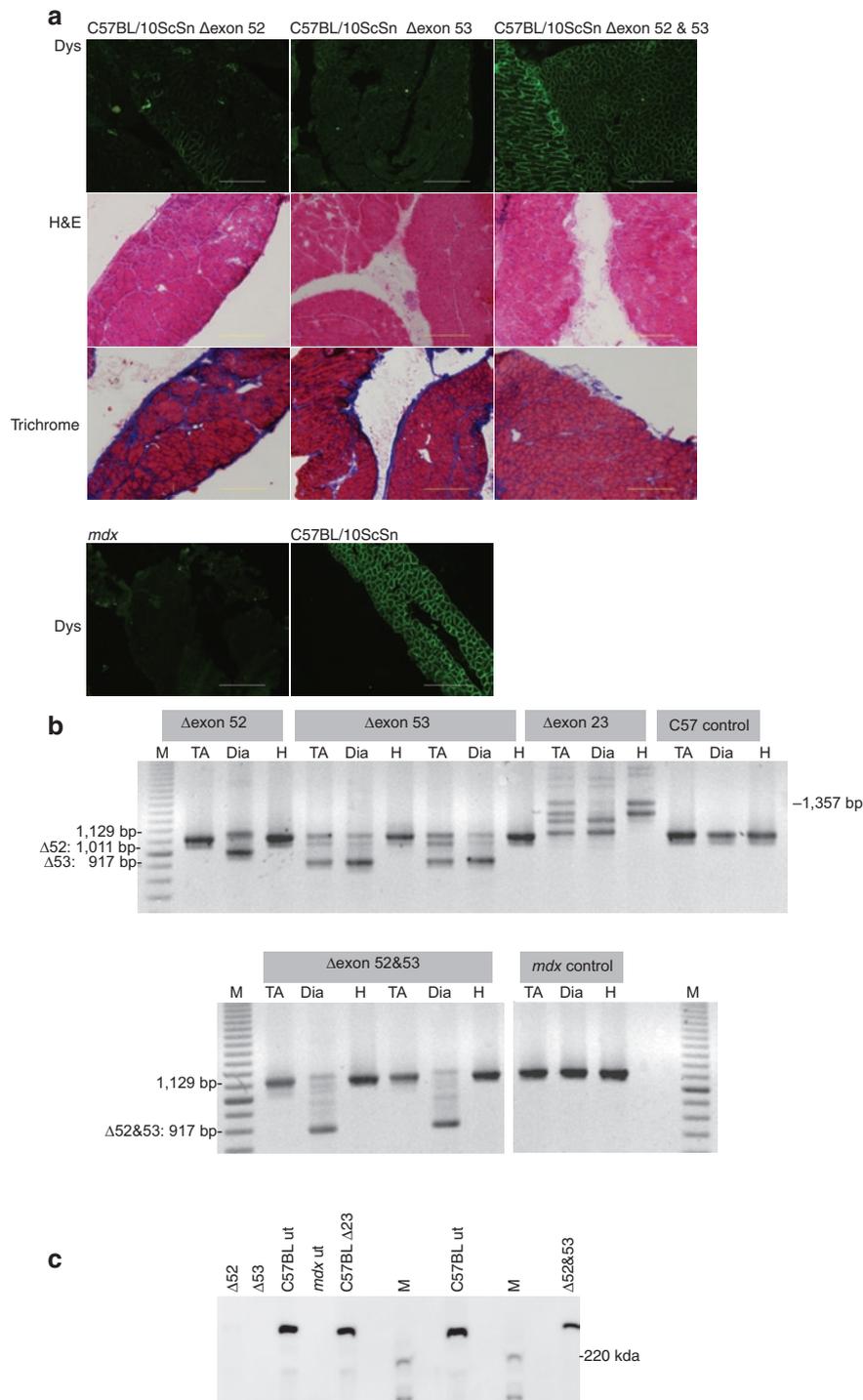
Figure 3 Analysis of dystrophin expression in C57BL/10ScSn mice treated with PMOs (20 mg/kg) targeting dystrophin 52 and 53. (**a**) C57BL/10ScSn mice were injected twice-weekly with peptide-conjugated PMOs (combined dosage 20 mg/kg) for 4 weeks, beginning at 4 days of age. Dystrophin expression in unfixed cryosections (6 µm) from diaphragm of C57BL/10ScSn mice treated with oligomers targeted to exon 52 (Δ52), exon 53 (Δ53), and exons 52 and 53 together (Δ52 and 53) was detected with Novocastra NCLDYS2 and Zenon Alexafluor 488 (upper panel). Sections from untreated, age-matched C57BL/10ScSn and *mdx* mice are included for comparison (lowest panel). Sections were also stained with haematoxylin and eosin (H&E) and Picro Mallory trichrome to reveal any pathogenic changes in muscle architecture (middle panels) (bar = 20 µm). (**b**) Nested RT-PCR across dystrophin exons 49–55 on RNA prepared from diaphragm (Dia), tibialis anterior (TA), and heart (H) of oligomer treated and untreated mice (M = 100 bp marker) and (**c**) western blot on extracts prepared from diaphragms of C57BL/10ScSn mice treated with oligomers targeted to exon 52 (Δ52), exon 53 (Δ53), and exons 52 and 53 together (Δ52 and 53). Dystrophin was visualized with NCLDYS2 (Novocastra) using Western Breeze (Invitrogen). Samples from diaphragms of C57BL/10ScSn (C57BL ut) and *mdx* mice (*mdx* ut) injected with vehicle only (saline), and a normal mouse, treated with an oligomer targeting dystrophin exon 23 (C57BL Δ23) are included for comparison (M = protein standard maker).

The human dystrophin deletion hotspot extends from exon 44 to exon 55. Antisense compounds to remove exon 51 are currently undergoing clinical trials, however, many additional compounds to exclude both single and multiple exons are required to address the majority of dystrophin mutations in this region. As in-frame deletions in this mutation prone region generally result in a BMD phenotype, we wished to demonstrate that exclusion of another dual exon block could result in functional dystrophin expression. As expected, exclusion of exons 52 and 53 induced near-normal levels of dystrophin expression and muscle architecture was indistinguishable from that of sham-treated normal mice. This technique will allow evaluation of dystrophin isoforms induced by selected exon removal, and in particular, will identify the optimal exon-skipping strategies for those mutations that may be addressed by more than one exon skipping approach. Similarly, bypassing a mutation involving exon 51 could be achieved by removal of either flanking exon, as dystrophin transcripts missing exon 50 and 51, or 51 and 52 are in-frame. Although both these dystrophin isoforms are associated with mild symptoms, it is possible one may be more functional than the other. Exons 50 and 51 encode the entire hinge 3, whereas part of hinge 3 is retained in the isoform missing exons 51 and 52.

It is also possible to efficiently disrupt the normal dystrophin mRNA reading frame by removing a frameshifting exon, and ablate dystrophin expression in the muscle of wild-type mice. Total suppression of *DMD* gene expression can be induced and maintained for several weeks *in vivo*, and a severe dystrophic pathology observed within 4 weeks of commencing treatment in neonatal normal mice. PMOs have been widely used to study gene knockdown by translational blockade,[36–38] however, induced nonproductive splicing provides a specific and effective alternative for the transient suppression of gene expression. Grounds *et al.*,[39] have proposed a two-tiered model of DMD, and suggest that the absence of dystrophin has a greater impact on growing muscle than it does on adult muscle. Further investigation in adult mice, using this strategy, may help to elucidate the impact of dystrophin disruption in mature muscle. Disruption of gene expression through altered splicing patterns could be applied across any part of the *DMD* gene transcript or to other genes, and offers the ability to induce transient animal models to study the consequences of gene suppression and splice-switching *in vivo*. This strategy would be particularly useful in studying the expression of genes that result in embryonic lethality when suppressed.

MATERIALS AND METHODS

In vivo *oligomer application.* C57BL/10ScSn mice were supplied by the Animal Resources Centre (Murdoch, Australia) and housed at the Biological Research Facility, University of Western Australia according to the National Health and Medical Research Council Code of Practice. All experiments performed on animals were approved by the University of Western Australia Animal Experimentation Committee (approval number RA4/100/702). C57BL/10ScSn mice were injected twice-weekly with peptide-conjugated PMOs[4] in normal saline (combined oligomer dosage of 20 mg/kg) targeting dystrophin exons as indicated, beginning at 4 days of age.

Antisense oligomers. PMOs targeting exon 19; H19A(+35+65), (GCCUGA GCUGAUCUGCUGGCAUCUUGCAGUU),[40] exon 20; M20A(+23+47),

(GUUCAGUUGUUCUGAAGCUUGUCUG) and M20A(+140+164), (AG UAGUUGUCAUCUGUUCCAAUUGU),[41] exon 23; M23D(+7−18), (GGC CAAACCUCGGCUUACCUGAAAT),[4,42] exon 52; M52A(+17+41), (UCCA AUUGGGGGCGUCUCUGUUCCA) and M52A(+42+71), (UUCAAAU UCUGGGCAGCAGUAAUGAGUUCU) and 53 M53A(+69+98) (CAGC CAUUGUGUUGAAUCCUUUAACAUUUC) and M53D(+05−25), (UU UUUAAAGAUAUGCUUGACACUAACCUUGG),[19] conjugated to peptide K[30] were supplied by AVI Biopharma (Corvallis, OR). Oligomers were diluted in saline and combined into cocktails at the following ratios: exons 19 and 20/20, 2:1:1, exon 52, 1:1, exon 53 1:1 and exons 52 and 53, 3:1, and applied at a total dosage of 20 mg/kg per injection.

Histology and immunofluorescence. Dystrophin expression on unfixed cryosections (6 μm) from muscles of C57BL/10ScSn, mice treated with oligomers, or sham injected, was detected with NCLDYS2 (Novocastra, Newcastle Upon Tyne, UK) and Zenon Alexafluor 488 (Invitrogen, Melbourne, Australia) according to the manufacture's instructions.

Western blotting. Western blot extracts were prepared from tissue cryosections and 7.5 μg of each sample was loaded onto 4–12% NuPAGE Novex BIS/Tris gradient gels (Invitrogen) and electrophoresed. The gels were stained and densitometry of the myosin band was used to standardize loading of the samples for a second gel that was blotted as described previously.[4,27] Dystrophin was visualized with NCLDYS2 (Novocastra) using the WesternBreeze Chemiluminescent anti-mouse Kit (Invitrogen). Images were captured on a Chemismart 3000 Gel documentation system (Vilber Lourmat, Marne-la-Vallee, France).

RNA extraction and RT-PCR. RNA was extracted from tissue cryosections using TRIzol Reagent (Invitrogen) and dystrophin transcripts were analyzed by nested RT-PCR across exons 18–26[8] and exons 49–55 for 35 and 30 cycles, as described previously.[19]

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