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Pre-mRNA splicing manipulation via Antisense Oligomers

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PRE-mRNA SPLICING MANIPULATION VIA ANTISENSE OLIGOMERS

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Abstract

Duchenne muscular dystrophy (DMD), the most common lethal neuromuscular disease in childhood, arises from protein-truncating mutations in the dystrophin gene. A deficiency in dystrophin leads to loss of the dystrophin associated protein complex (DAPC), which in turn, renders muscle fibres vulnerable to injury, and eventually leads to muscle loss, necrosis and fibrosis.

Although, the dystrophin gene was identified nearly two decades ago, and extensive research has been directed at finding a therapy for DMD, to date, there is still no effective treatment available. One promising molecular approach to treat DMD is antisense oligomer (AO) induced splice intervention. AOs were most widely used to induce RNaseH-mediated gene transcript degradation, however, the development of different backbone chemistries heralds a new generation of AOs that can modify gene transcript splicing patterns. Application of AOs to the dystrophin pre-mRNA to influence exon selection and induce shortened, in-frame dystrophin isoforms is being vigorously pursued.

The majority of the work presented here explores the concept of personalised therapies for DMD, whereby oligomers are designed to specifically target individual mutations. The importance of AO-optimisation to obtain AOs capable of inducing efficient dual exon skipping in an established animal model of muscular dystrophy (4^{CV} mouse), which carries a DMD-causing mutation in exon 53, is demonstrated. Removal of both exons 52 and 53 was required to by-pass the mutation, maintain the reading frame and restore dystrophin expression.

One of the major challenges of AO-induced splice intervention for therapeutic purposes will be the design and development of clinically relevant oligomers for many different mutations. Various models, including cells transfected with artificial constructs and mice carrying a human dystrophin transgene, have been proposed as tools to facilitate oligomer design for splice manipulation. This thesis investigates the relevance of using mouse models to design AOs for human application, and also explores the use of cultured human myoblasts, from both unaffected humans and a DMD patient, as a means of establishing the most effective therapeutic compound.

In addition to induction of exon skipping, the applicability of AOs to promote exon inclusion, by masking possible intronic silencing motifs of survival motor neuron (SMN) pre-mRNA in cultured fibroblasts from a spinal muscular atrophy (SMA)

patient, is investigated. This study provides additional information about a novel oligomer target site that could be used in combination with previously identified splice silencing motifs for a molecular therapeutic approach to SMA, and may perhaps open up new avenues of treatment for other genetic disorders, where oligomers could be used to induce exon inclusion.

PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

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Conference Poster presentations

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ABBREVIATION

2'OMeAO	2'-O-Methyl modified sugars on phosphorothioate backbone
4^{Cv} mouse	B6Ros.Cg-Dmd$^{mdx\text{-}4Cv}$/J mouse
AAV	Adeno-associated virus
Ab	Antibody
Acta1	α-actin gene
ADP	Adenosine diphosphate
AO	Antisense oligomer
ATP	Adenosine triphosphate
bcl-2	B cell chronic lymphocytic leukemia/lymphoma 2 protein
BMD	Becker muscular dystrophy
cDNA	Complementary deoxyribonucleic acid
CK	Creatine kinase
c-myc	Myelocytomatosis oncogene
CNND	Centre for Neuromuscular and Neurological Disorders
CNS	Central nervous system
CPP	Cell
CTD	c-terminal domain (of RNA polymerase II)
DAPC	Dystrophin associated protein complex
DHPR	Dihydropyridine receptor
DMD	Duchenne muscular

DMEM	Dulbecco modified Eagle's medium
DNA	deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
$\Delta\psi$	Electrochemical mitochondrial membrane potential
EBV	Ebstein-Barr virus
EDI exon	Extra domain 1 exon
EJC	Exon junction complex
ENA	4′-C-ethylene bridge nucleic acid
ES	Embryonic stem cell
ESE	Exonic splice enhancer
ESS	Exonic splice silencer
ESTs	Expressed sequence tags
FCS	Foetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
GRMD	Golden retriever muscular dystrophy
H-2K mdx	*H-2K*^{b}-tsA58 *mdx* myoblasts
hnRNP	Heterogeneous nuclear ribonucleoprotein
HRP	Horse raddish peroxidase
HS	Horse serum
HSV	Herpes simplex virus

IGF-1	Insulin like growth factor 1
ISE	Intronic splice enhancer
ISS	Intronic splice silencer
kb	Kilobase
kDa	Kilodalton
LGMD	Limb girdle muscular dystrophy
LNA	Locked nucleic acid
Mb	Megabase
MDA	Muscular dystrophy association
MEM	Maximum entropy model
miRBASE	the microRNA database
miRNA	microRNA
MM	First-order Markov model
MOE	2'-O-methoxy ethoxy modified sugars on phosphorothioate backbone
MPC	Muscle precursor cell
MPTP	Mitochondrial permeability transition pore
mRNP	messenger ribonucleoprotein
Mw	Moleculat weight
Myh7	Myosin heavy chain-β gene
nM	Nanomolar
NMD	Nonsense mediated decay
NMJ	Neuromuscular junction

nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
Nppa	Atrial natriuretic factor gene
ORF	Open reading frame
PCR	Polymerase chain reaction
PMO	Phosphorodiamidate morpholino oligomer
PNA	Peptide nucleic acid
PO	Phosphodiester
pre-mRNA	precursor messenger ribonucleic acid
prp	pre-mRNA splicing factor RNA helicase protein
PS	Phosphorothioate
PTC	Premature termination codon
QEII	The Queen Elizabeth II medical centre
RDOs	Chimeric RNA/DNA oligonucleotides
RGG	Arginine-Glycine-Glycine tripeptide
RNA	Ribonucleic acid
RNAi	RNA interference
RNAP II	RNA polymerase 2
RNaseH	RNase H enzyme
ROS	Reactive oxygen species
RRMs	RNA recognition motifs
RS domain	arginine/serine dipeptide rich domain

RT	Reverse transcription
RYR	Ryanodine receptor
SCID	Severe combined immuned deficiency
SF2/ASF	Splicing factor 2 and Arginine/Serine rich-1 protein complex
siRNA	Short interference RNA
SMA	Spinal muscular atrophy
SMN	Survival motor neuron gene
snRNA	Small nuclear ribonucleic acid
snRNP	Small nuclear ribonucleoprotein
SOCs	Store operative calcium channels
SR protein	Serine/Arginine rich protein
TA	Tibialis anterior
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrotic factor-α
U2AF65	The 65 kDa subunit of U2 small nuclear RNA auxiliary factor 2
UTR	Untranslated region

CHAPTER 1

Introduction

1.1 Muscle biology and the role of dystrophin

Skeletal muscle, which accounts for approximately 30-40% of human bodymass,[1] is composed of myofibres wrapped within a cell membrane termed the sarcolemma. Myofibres comprise several hundreds of myofibrils, which are surrounded by the highly organised sarcotubular system, mitochondria, and
Stratified layers of actin and myosin filaments and other proteins (including tropomyosin, members of the troponin family, calpain-3, and titin) form the myofibrils, which are subdivided by the Z plate (Z-line) into 2μm-long contractile units called sarcomeres.

Figure 1 illustrates the sti
contraction is stimulated. Muscle contraction begins after the transmission of a stimulus from the motor neuron to the motor end plate embedded in skeletal muscle (**Fig 1a**). Muscle-membrane action potentials, which are generated by acetylcholine-stimulated cation channels on the sarcolemma, excite voltage-sensitive dihydropyridine receptors (DHPRs) located in the "triad" region (**Fig 1b**). Once activated, DHPRs alter their conformation resulting in the release of calcium ions from ryanodine receptors (RYRs) residing on the sarcoplasmic membrane.

The presence of calcium ions in conjunction with ATP activates the myosin calcium-dependent ATPase. Detachment of inorganic phosphate from the complex leads to a conformational change in the head of the myosin molecule, increasing the affinity between actin and myosin filaments, causing the sliding movement of myosin along the actin filaments. Release of ADP brings the myosin head into its final position. Subsequently, the recruitment of a new ATP molecule loosens the actin-myosin association and as a consequence the myosin head moves back to its erect position. Contractile forces on each sarcomere lead to muscle contraction with the assistance of Z-plates, M-p
important in the context of this thesis,
cytoskeletal actin to extracellular matrices in the muscle fascicle during contraction and relaxation, through the dystrophin-associated protein complex (DAPC).

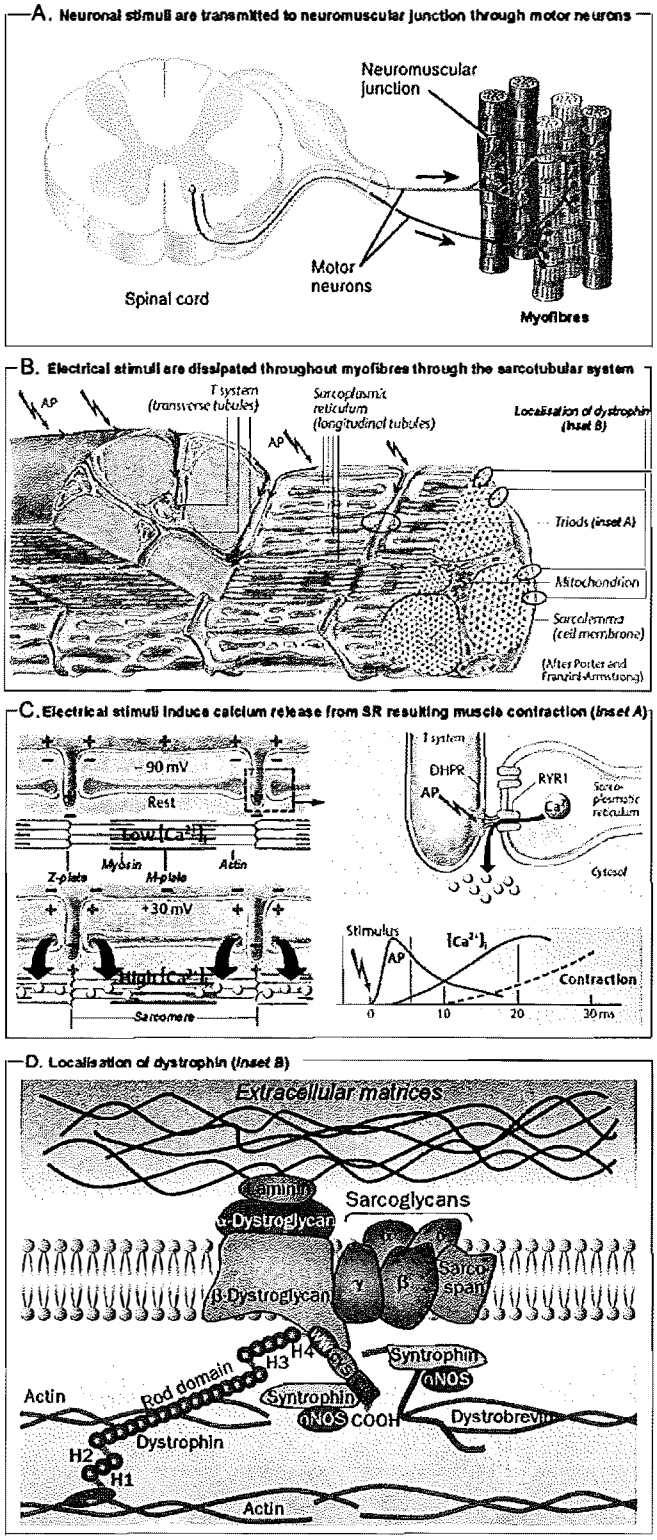

Figure 1: Muscle contraction and the role of dystrophin.

The phases of contraction shown are (a) neuronal stimuli from anterior horn cells are transmitted to the neuromuscular junction; (b) action potentials (APs) on the sarcolemma generated by the release of the acetylcholine from the presynaptic membrane, are dissipated throughout the myofibre through the sarcotubular system (T system); and APs activate the discharge of calcium ions from the sarcoplasm (SR); and (c) release of calcium ions induce contraction of the sarcomeres, which are primarily composed of two major contractile proteins, actin and myosin; (d) inset B shows the localisation of dystrophin in the myofibre and its positional relationship to the actin cytoskeleton and extracellular matrices (modified from Daspopoulos *et al.* 2003,[2] Kapsa *et al.* 2003[3]).

1.2 Dystrophin, its role in muscle biology and its molecular and genetic complexity

The dystrophin gene has an exceptionally large 2.5 Mb genomic sequence,[4] encoding a 14 kb full-length transcript[5] derived from a total of 79 exons,[6] (**Fig 2**) which in turn translates into a 427 kDa protein. At least seven, possibly eight promoters, spanning the dystrophin gene are known to regulate tissue-specific expression of dystrophin (**Fig 3a**). The structural features of the various dystrophin isoforms are illustrated in **Figure 3b**. Four full-length 427 kDa isoforms are present in muscle, lymphocytes, brain (neurons), and Purkinje cells.

Dystrophin has been identified as having four major domains, an amino terminal domain, a 24 helical spectrin-like repeat rod domain containing four hinge regions, a cysteine-rich domain, and the carboxy terminal domain (**Fig 2**). The amino terminal domain of dystrophin interacts with the actin filament of myofibrils. The hinged structure of the spectrin-rich 2,839 amino acid rod domain, which contains a secondary actin binding site, provides structural flexibility and allows the dystrophin molecule to retain functional integrity in the face of the sheer forces associated with the contractile process in muscle.[7] The cysteine rich domain, comprising a WW domain,[8] EF hand-like motifs,[9] and ZZ domains,[10] binds to β-dystroglycan, forming the DAPC (**Fig 1d**). The carboxy terminal domain is responsible for the docking of α-syntrophin and dystrobrevin within the sarcoplasm. The α-syntrophin associated with neuronal nitric oxide synthase (nNOS) functions in anchoring ion channels and signalling molecules.

1.3 Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD), the most common neuromuscular disorder of young adults affecting 1 in 3,500 boys, is caused by the ablation of functional dystrophin, rendering muscle more susceptible to membrane damage. During infancy, affected DMD patients appear normal but show delayed walking and experience difficulties in running and climbing in early childhood. As the disease progresses, weakness in the knee and hip extensor muscles becomes conspicuous and results in a waddling gate and difficulties in rising from the floor (Gower's manoeuvre). DMD patients thereafter lose their ambulation around the age of 12. Although, the use of assisted ventilation reduces hospitalisation time and prolongs the life expectancy of affected DMD boys by approximately 10 years,[11, 12] by their late twenties, patients

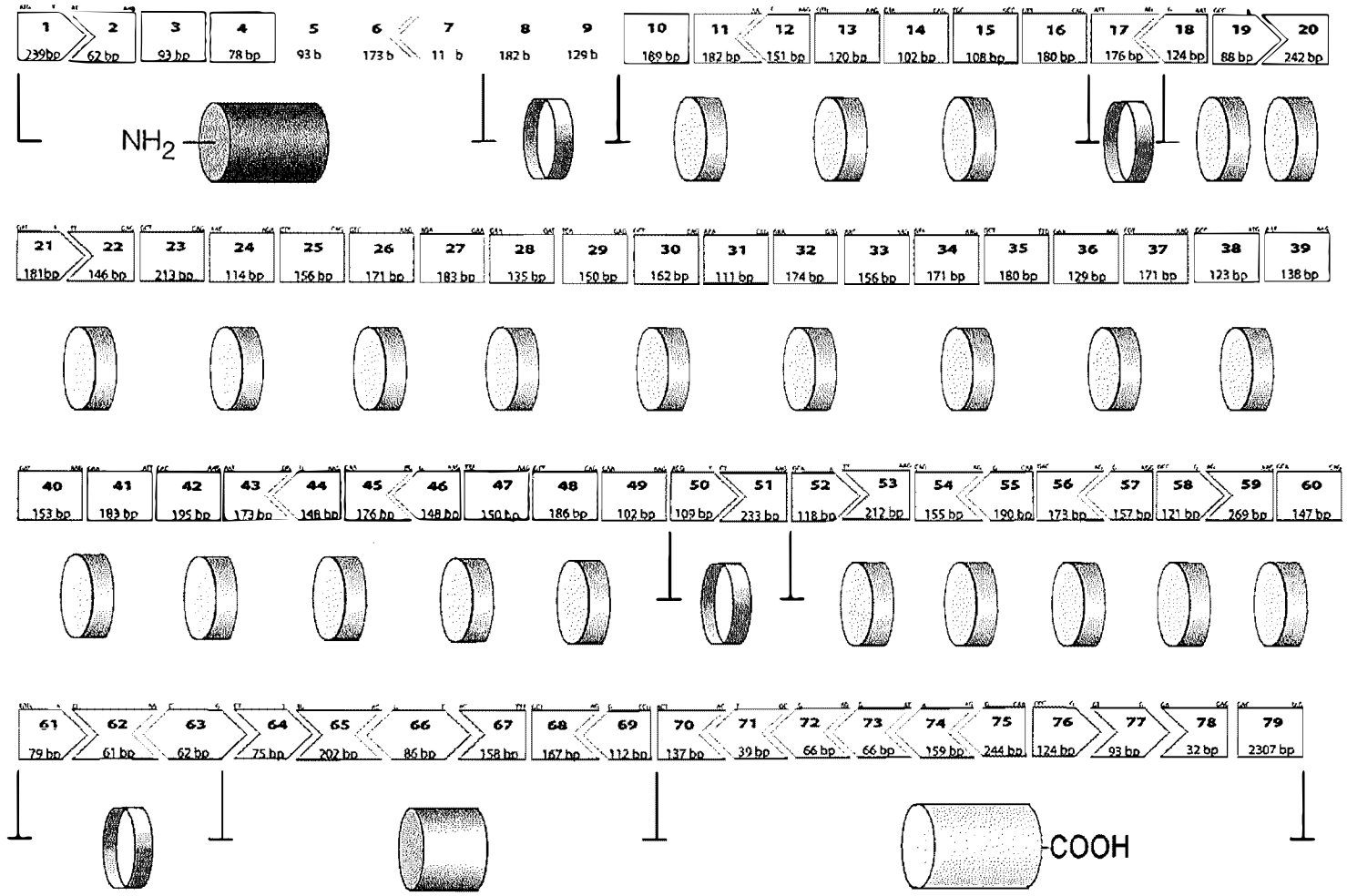

Figure 2: Layout of dystrophin cDNA and the encoded functional domains of dystrophin protein.

The figure shows the alignment of the four functional domains of dystrophin separated by vertical lines (viz. amino terminal (red cylinder), 24-spectrin repeat rod domain (blue cylinders) with four hinges (teal rings), cysteine-rich domain (green cylinder), and the c-terminal domain (orange cylinder)) to the cDNA encoded by the 79 exons of the dystrophin gene. The size of the exons and the triplet codons on either side of the exon junctions are indicated (not shown to scale) (modified from van Deutekom *et al.* 2003[11]).

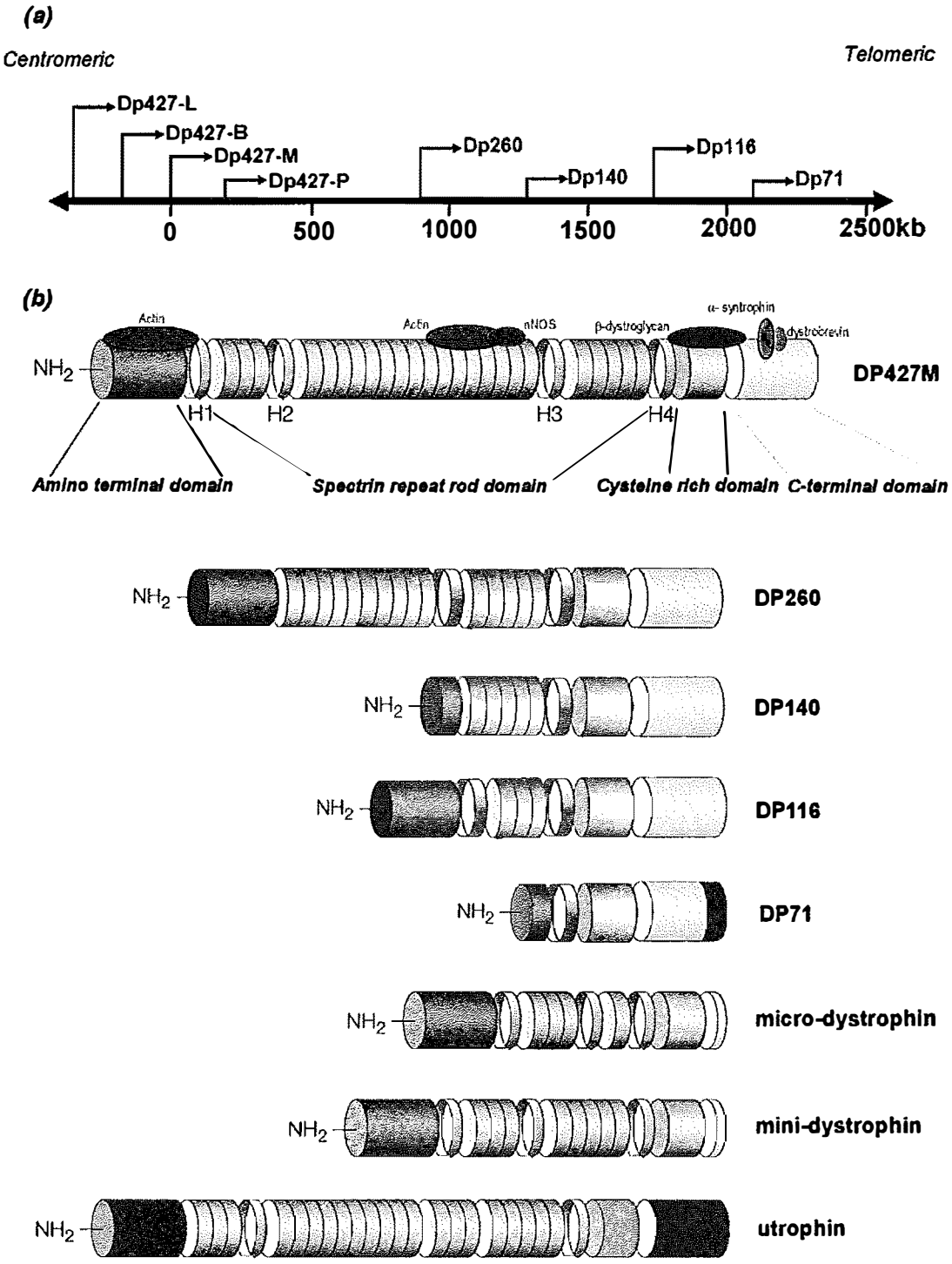

Figure 3: Organisation of the dystrophin gene, protein and homologues.

(a) The structure of the dystrophin gene and the location of its eight promoters, which lead to the expression of multiple isoforms (viz. Dp427-L, Dp427-B Dp427-M Dp427-P, Dp260, Dp116, Dp140, Dp40, Dp71) in different cell types, depending on which promoters are operational, and (b) the structure of five major isofo ms of dystrophin, micro-dystrophin, mini-dystrophin and the dystrophin homologue, utrophin. The four functional domains of the protein and the regions where dystrophin interacts with other proteins are illustrated (modified from Sadoulet-puccio *et al.* 1996[11] and van Deutekom *et al.* 2003[12]).

typically succumb to respiratory failure or cardiac complications.

The absence of dystrophin leads to a failure to assemble the DAPC, resulting in chronic membrane injuries promoting calcium ion leakage. After exercise, more calcium ions are pumped into the cytosolic space through mechano-sensitive cationic receptors and store operative calcium channels (SOCs).[15-17] Calcium influx induces calcium release from the sarcoplasmic reticulum[18] and also activates the protease calpain, causing protein degradation. A sustained surge of cytosolic calcium increases intra-mitochondrial calcium levels, thus opening the mitochondrial permeability transition pore (MPTP), which, in turn, dissipates the electrochemical mitochondrial membrane potential ($\Delta\psi$) and pH gradient. The consequent loss of the proton motive force leads not only to disrupted ATP production because of impaired oxidative phosphorylation, but also promotes cytosolic ATP breakdown. In addition, the rise in intra-mitochondrial calcium stimulates reactive oxygen species (ROS) production through inhibition of electron transport. These pathological changes eventually lead to necrotic cell death through the activation of phospholipases, nucleases, and proteases and to muscle cell death, inflammation, and fibrosis (for review see[19-25]).

1.4 Becker muscular dystrophy and the reading frame rule

A milder allelic variant, Becker muscular dystrophy (BMD), has a broader and commonly less severe spectrum of clinical symptoms, depending on the size and location of the mutation. There is no simple correlation between the extent of the genetic lesion and phenotypic severity. Indeed, although both DMD and BMD carry mutations of the same gene, it is the disruption of the dystrophin reading frame in DMD that is responsible for the severity of the symptoms in DMD. These observations have primarily led to the reading frame rule, which states that the severity of symptoms in DMD as opposed to BMD is the direct result of disruption of the reading frame.[26] Koenig[27] (1989) has reported that the rule holds in 90 to 95% of dystrophinopathy cases.

DMD patients with in-frame mutations usually carry large deletions in the 5' region, which presumably causes compromised actin binding.[28-31] Although the in-frame deletions in the repeat rod domain are usually benign,[32, 33] this is not the case in patients who have lost more than 35 exons from the dystrophin gene.[30]

Some deletions at the genomic DNA level can affect pre-mRNA splicing, inducing naturally occurring exon skipping. RT-PCR analysis of RNA from patient myoblasts has detected in-frame alternatively spliced transcripts missing exons 2-7 (Δ2-7) and exons 3-8/9 (Δ3-8/9).[34] This may explain why patients carrying a deletion of exons 3-7 (Δ3-7) have a milder phenotype. Another possible reason for the milder than expected phenotypes of BMD patients is the use of an alternative translation initiation codon located in exon 8.[35-37]

It is important to recognise that most studies, until late 90s, have involved diagnosis at the genomic DNA level, which can lead to diagnostic confusion. In fact, over 99.5% of dystrophinopathy patients fit the reading frame rule if diagnosis involves analysis at the DNA, RNA and protein levels.[38] For this reason, it is now recommended that diagnosis based on genomic DNA is confirmed ideally by diagnosis at the RNA and protein levels.[38]

1.5 Approaches to the treatment of DMD

During the more than twenty years since the mapping and isolation of the dystrophin gene, various approaches have been explored to ameliorate the symptoms of DMD or forestall their onset. There are three basic therapeutic approaches to the treatment of DMD, indirect therapies, direct therapies and personalised therapies.

1.5.1. Indirect therapies

Indirect therapies involve the modulation of other genes or proteins involved in the pathogenesis of DMD. Therapies include enhancement of utrophin expression, chronic steroid administration, use of TNF-α inhibitor and use of a myostatin inhibitor.

Upregulation of the dystrophin homologue, utrophin, a foetal form of dystrophin encoded by a separate gene, whose expression is abundant only at neuromuscular and myotendinous junctions after birth,[39-42] creates a situation where utrophin is able to partially assume the function of dystrophin. The A isoform of utrophin, whose expression is under the control of promoter A, has been observed at the sarcolemma of *mdx* mice and DMD patients. Molecular characterisation of promoter A has been undertaken, with the goal of identifying transcriptional motifs which may potentially be targets for utrophin upregulation.[43] The nerve growth factor, heregulin, stimulates utrophin expression through its binding to a transcriptional motif.[44] Increasing levels of

utrophin protein three-fold is reportedly sufficient to ameliorate the dystrophic phenotype after intraperitoneal injection of recombinant heregulin in *mdx* mice.

Mattei *et al.*[45] (2007) produced VP-16 jazz zinc finger protein transgenic mice with upregulated utrophin expression via promoter A enhancement and showed that increased utrophin levels led to stabilisation of the DAPC. Increasing utrophin levels by systemic delivery viral constructs expressing this zinc finger protein as opposed to transgenesis and applying the technique to larger animals are issues that need to be resolved before the technique can be applied more broadly.

A second indirect therapy, chronic systemic steroid administration has been shown to increase muscle functionality, improve respiratory function and decrease respiratory complications in DMD patients.[46, 47] Although clearly providing benefit to DMD patients, chronic oral steroid use also leads to adverse effects, including excessive weight gain, behavioural problems, insomnia, reduction in bone density and compromised immunity. An anti-inflammatory effect[48] coupled with promotion of proliferation and/or fusion of muscle precursor cells[49] is thought to be the mechanism underlying the amelioration of muscle functionality in *mdx* mice by steroids.

While steroid administration improves the pathology of DMD through several mechanisms, blocking the activity of the important pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), provides another option. Treatment with Infliximab, the neutralising antibody of TNF-α, has been shown to reduce muscle necrosis in young *mdx* mice[50, 51] and exercised adult mice.[52]

Administration of a myostatin inhibitor in combination with steroids or direct therapy provides a mechanism to boost general muscle mass and enhance muscle differentiation. Long-term inhibition of myostatin might however accelerate the depletion of muscle regenerative capacity in the setting of a chronic muscle disease.[53]

1.5.2. Direct therapies

Dystrophin replacement therapy, which involves direct administration of dystrophin (or dystrophin homologue) cDNAs or cells expressing dystrophin, has been extensively studied using both gene replacement and cell therapy techniques. Both viral and non-viral approaches have been utilised as tools for the delivery of dystrophin cDNA. Analysis of mutations in BMD patients suggests that a major part of the gene

can be excluded with minimal effect on function. cDNAs of mini-dystrophin (6.3kb) and micro-dystrophin (4.8kb) (**Fig 3b**) have been characterised and successfully utilised as a dystrophin replacement therapy for DMD patients, although their use is severely limited by the associated adverse immune response to the viral vectors.

Plasmid DNA inducing full length dystrophin has been demonstrated to be present and partially restore dystrophin protein levels in rodent, canine and non-human primate models of muscular dystrophy after its systemic administration, with a minimal adverse immune response. However, the comparatively low efficiency of this technique is a major drawback.[54, 55]

Treatment with the adeno-associated virus (AAV) constructs, linked to micro-dystrophin cDNA, has been shown to lead to systemically increased levels of dystrophin expression in *mdx* mice.[56, 57] However, this approach is problematical because it provokes an immune response limiting the option for subsequent treatment. Two different laboratories using similar approaches have reported adverse immune responses against viral constructs of AAV2 and AAV6 carrying either a reporter gene or micro-dystrophin.[58, 59] In addition to an adverse immune response, viral approaches may potentially lead to multiple integrations, insertional mutagenesis, or proliferative disorders.[60]

Cell therapy involves the administration to patients of adult stem cells, such as muscle precursor cells (MPCs) or mesangioblasts carrying a normal dystrophin gene. These stem cells can come from human donors with normal dystrophin (heterologous stem cells) or involve the use of the patient's own stem cells (autologous stem cells) treated to augment dystrophin protein levels. Quenneville *et al.*[61] (2007) have reported successful intramuscular autologous transplantation of lentiviral vector microdystrophin-treated MPCs in *mdx* mice and non-human primates in a protocol incorporating immunosuppressant therapy. Recently, use of a high density injection protocol of MPCs was shown to enhance dystrophin expression and lead to functional improvement 18 months after intramuscular injection in *mdx* mice.[62]

Another cell type, heterologous mesangioblasts intra-arterially administered to immunosuppressed dystrophic dogs, has been shown to improve muscle functionality.[63] The major challenges of cell therapy are avoiding sustained immunosuppression and ensuring the systemic delivery of stem cells. The transplant of autologous stem cells genetically corrected by viral constructs would inevitably share similar limitations to

viral gene replacement strategy.

1.5.3. Personalised therapies

Mutation-specific therapies, a form of personalised medicine, provide an alternative approach to DMD molecular therapy. A prerequisite is that patient mutations must first be characterised. The different targets for correction include gene editing or mRNA modulation. Gene editing could theoretically correct both nonsense and frameshift mutations, however, only nonsense mutations of *mdx* and *mdx*^{5cv} mice have been proven to restore reading frame functionality.[64, 65] Gene editing could also be used in genetically-modified autologous stem cell therapy.[66]

High concentrations of aminoglycoside antibiotics, such as gentamycin, under some circumstances are known to force the translational machinery to read through stop codons. Up to twenty percent of dystrophin transcripts were restored after gentamycin treatment in *mdx* mice.[67] A subsequent study using gentamycin in DMD and BMD patients, however, did not produce such an encouraging result,[68] possibly because multiple isoforms of gentamycin cause variations in potency. Identification of small molecules, which specifically suppress UGA nonsense codons, has been examined using high-throughput drug screening. PTC124 was demonstrated to read through UGA with minimal adverse effects, and to partially restore dystrophin levels (as determined by immuno-staining) and delay disease progression in *mdx* mice following a combination of oral and intraperitoneal administration.[69] Both gene editing and PTC 124 treatment in theory could be applied in the 10-15% of DMD patients who carry a nonsense mutation.

1.6 Principles underlying the antisense oligomer induced exon skipping approach

Until now, because many different approaches to develop molecular therapies for DMD all have some limitations, there has been a lack of consensus as to the best therapeutic strategy. Pre-mRNA splice intervention using antisense oligomers (AOs) potentially offers a personalised approach to therapy where AOs can efficiently induce functional dystrophin with minimally provoked adverse immune responses, and theoretically be applied to the majority of DMD patients.

The AO-induced exon skipping concept was initially put forward because some large deletions of the dystrophin gene cause only mild symptoms in some cases of BMD. In addition, rare dystrophin-positive revertant fibres have been shown to arise by a naturally occurring exon skipping mechanism. At the molecular level, the significant differences between DMD and BMD are due to the different extents of reading-frame disruption.[26] Consequently, it has been proposed that AOs could help DMD patients by dislodging some exons to circumvent reading-frame disruption, thus restoring functional dystrophin expression and re-establishing the sarcolemmal protein complex.

Although the ultimate goal of using oligomers is to interfere with pre-mRNA splicing by preventing exon recognition and selection,[70, 71] the challenge is that splicing is tightly linked to transcription. Understanding the flow of genetic information (viz. transcription, splicing, 5′ capping, 3′ polyadenylation, mRNA export and translation) could potentially improve oligomer design for exon removal both in DMD and other genetic diseases and broaden the application of oligomers to other processes in gene expression.

1.7 Gene expression and splicing

1.7.1 The complexity of gene expression and pre-mRNA splicing

Transcription is the synthesis of RNA from a DNA template. For a protein-coding gene, RNA polymerase II (RNAP II) initially synthesises a full-length complementary RNA molecule, described as a nascent transcript or precursor mRNA (pre-mRNA) from the template strand. Eukaryotes exhibit greater genomic complexity than prokaryotes because intronic sequences are inserted between the coding sequences (exons). Consequently, the pre-mRNA needs to be modified by intron removal, before its commitment to the protein translational machinery. After RNA processing, the mature mRNA acts as a template for protein translation using groups of three nucleotides (triplet codons).[72]

Differential splicing of 5′ or 3′ splice sites either within the original exon or at cryptic splice junctions potentially allows individual genes to express multiple mRNAs that encode proteins with diverse functions. Seven types of alternative splicing mechanisms are recognised as shown in **Figure 4**. On the basis of conservative analyses of expressed sequence tags (ESTs) and cDNA datasets, alternative splicing has been reported to occur in some 40% to 60% of human genes, a figure that increases to

73% when splicing data is combined with information derived from alternative splicing microarray analysis.[73]

1.7.2 Splicing motifs and trans-factors

Pre-mRNA splicing is controlled by a battery of proteins (trans-acting factors), which act in combination to recognise specific sequence motifs (cis-acting elements) on pre-mRNA.[74] Both small nuclear ribonucleoproteins (snRNPs) and non-small nuclear ribonucleoproteins (non-snRNPs) cooperatively define the exon/intron border. SnRNPs are proteins tightly bound to a single uridine-rich small nuclear RNA (snRNA) and five snRNPs are involved in splicing (viz. U1, U2, U4, U5, and U6).[75] Non-snRNP splicing factors comprise an assortment of functionally diverse proteins that play a pivotal part in alternative splicing.[76] Non-snRNPs, which are specifically attached to pre-mRNA or to one another to enhance exon inclusion or exon repression, comprise two groups of proteins, serine-arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs).

Many members of the SR protein family have been demonstrated to possess an exon inclusion function.[77, 78] SR proteins contain one or two RNA recognition motifs (RRMs), which bind to pre-mRNA on the exonic sequence enhancer (ESE). They have a distinctive carboxy terminal domain that is rich in arginine/serine dipeptides (the RS domain). The RRMs determine substrate specificity via the ESE, whereas the RS domain is mainly involved in protein-protein interaction by directly recruiting the splicing machinery.[78]

Whereas SR proteins act by enhancing exon inclusion, hnRNPs act by inducing exon repression. The hnRNPs contain RRMs and protein K homology RNA binding motifs (KH domains), and RGG (Arg-Gly-Gly) boxes.[79] HnRNPs not only exhibit RNA-binding properties but also contain one or more protein-protein interacting domains.[76] Moreover, HnRNPs take part in various nuclear events, such as transcriptional regulation, pre-ribosomal RNA processing, 3′ end processing, and nuclear-cytoplasmic mRNA transport.[76] In summary, the aggregations of non-snRNP proteins attached to a cis-acting element in a spliceosome (see Section 1.8.2) define the exon/intron border and determine whether the exon is incorporated into transcript, with the snRNPs signalling the precise locations for splicing.

1.7.3 Spliceosome

Splicing was first discovered when Berget *et al.*[80] (1977) identified a splice segment in adenovirus. Shortly thereafter, a number of cellular genes in a variety of organisms were discovered to contain introns, including the rabbit and mouse globin genes,[81, 82] and mouse immunoglobulin.[83] Evolutionary analysis revealed a limited set of conserved sequences at each intron boundary.[84] These conserved sequences were shown to be common across vertebrates, plants, and yeasts.[85]

It is now recognised that the process of gene splicing involves an assembly of proteins, the spliceosome. The components of spliceosomes and their specific roles in splicing have been progressively identified in higher eukaryotes since the discovery of splicing in the 1970s.

The components of the spliceosome vary between different genes and even within the same cell type at different phases of gene expression, although there are five common snRNPs in every spliceosome. Both snRNPs and non-snRNPs perform an intron/exon defining function, and snRNP complexes are responsible for excising intervening sequences. Non-snRNPs are extensively involved in determining the existence of exons in certain transcripts.[78] Many methods have been utilised to determine the human spliceosome components,[75, 86] and a proteomic analysis of the human spliceosome has revealed the presence of 145 distinct proteins.[87] For this reason, the spliceosome is regarded as one of the largest and most complex macromolecular assemblies present in cells.[88]

1.7.4 Spliceosome assembly and the fidelity of pre-mRNA splicing

Spliceosomes form the dynamic complex responsible for splicing, a process which involves two successive esterification reactions (**Fig 5**). With a single catalytic core, dynamic conformation and multiple rearrangements are required to enable the two consecutive reactions to proceed. More than a decade ago, RNA helicases and members of the DExH/D-box ATPase family were proposed to have roles in spliceosome function and determine the fidelity of splicing, and have been directly demonstrated to unwind duplex RNA,[89] dissociate protein from RNA[90] and bind protein to RNA,[91] in an ATP-dependent manner. Subsequently, various RNA helicases, including prp28, prp16, prp5, prp 8, prp22, prp19 and prp43, have been suggested to control the repositioning of the spliceosomal catalytic core by inducing a conformational shift during the two

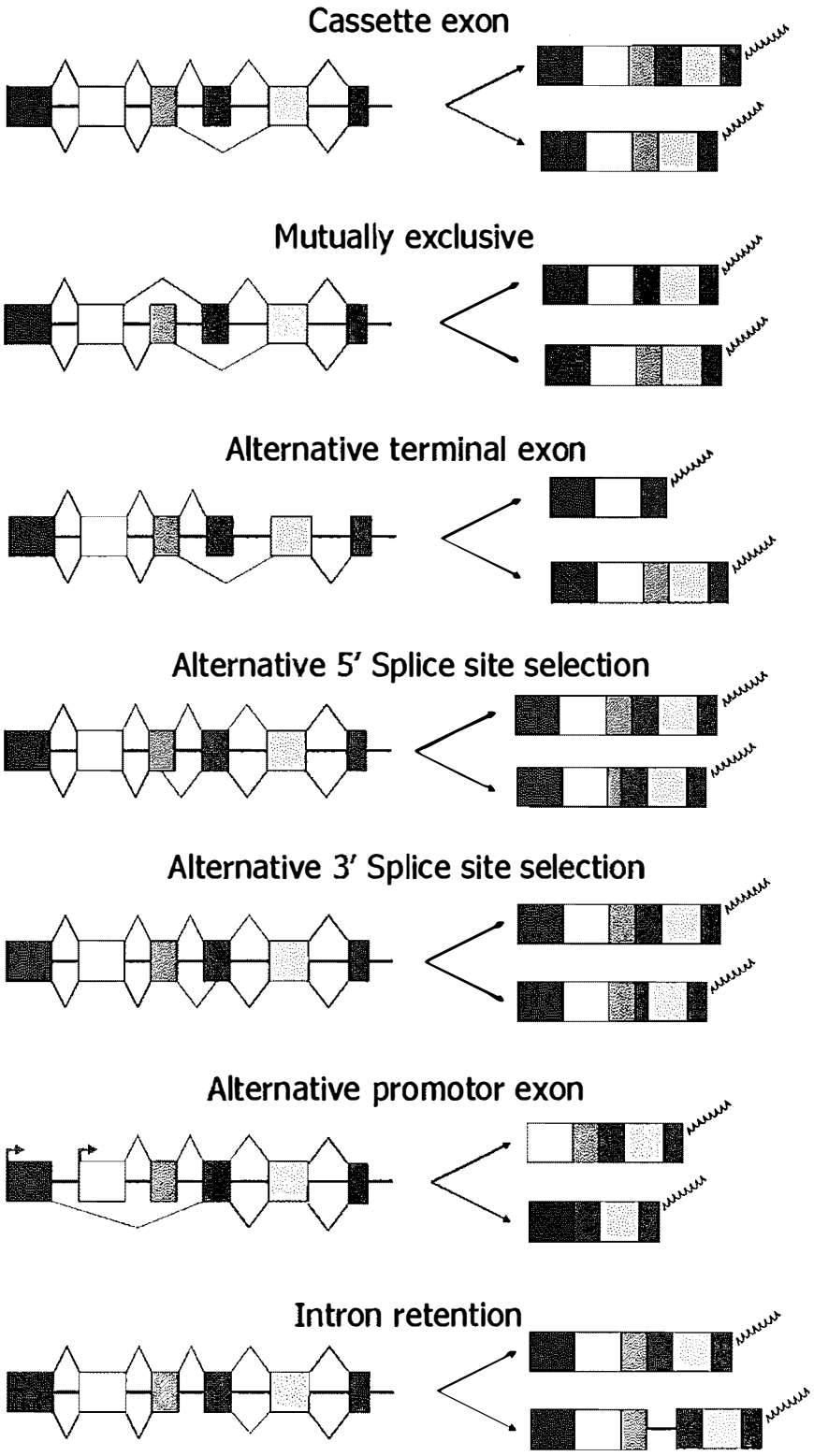

Figure 4: Patterns of alternative gene splicing.

The seven different types of alternative gene transcript splicing are illustrated. The coloured rectangles represent exonic regions, and the bold lines indicate the intronic regions. The blue lines represent the regions which are spliced out to produce the mature transcripts resulting from the seven different types of pre-mRNA splicing.

catalytic reactions, which allows spliceosomes to kinetically proofread the splice motif during pre-mRNA splicing (for review see [92]). **Figure 6** illustrates the conformational changes in the spliceosome during the two successive esterification reactions.

As illustrated in **Figure 5**, during assembly the U1 snRNP complex binds to the 5′ splice site via 6-7 base pairing between the splice site and U1 snRNA.[93] A branch point binding protein, SF1 binds to a branch point and the 65-kDa subunit of the U2/AF protein recognises a pyrimidine tract. The 35kDa subunit of U2/AF then attaches to the 3′ splice site.[93] Either direct RS-RS domain interaction or indirect interaction through splicing co-activators is required to ensure the recruitment of the splicing factor U2/AF65-kDa, especially in those cases in which recognition of a weak pyrimidine tract is a rate-limiting step.[77, 94]

At this stage, the assembly of proteins is referred to as complex E (**Fig 5**). Subsequently, U2 snRNPs are recruited to interact with complex E. U2 snRNPs attach to a branch site via U2 snRNA, which forms a stem IIa structure, thereby forming complex A.[93, 95] The A complex is joined by the U4/5/6 tri-snRNP to form the B complex. Brr2, a helicase protein, facilitates unwinding of the U4/U6 duplex, allowing U6 to displace U1 snRNA.[96] The coupling reaction of U1 displacement and U6 base-pairing to the 5′ splice site is mediated by phosphorylated prp28.[97-99] In conjunction with rearrangement of U2snRNA from stem IIa to stem IIc through prp5p and cus2p interaction, U6snRNA anneals to a region of U2snRNA that is near to the branch site duplex bulging 2′ hydroxyl group of adenosine.[100-103] Bulging of the 2′ hydroxyl group at the branch point base leads to nucleophilic attack at the 5′ splice site, and causes the first esterification.

After the first esterification, a prp16-dependent interaction coupled with ATP hydrolysis juxtaposes the 3′ splice site and the 5′ splice site, allowing progression to the second esterification step. This causes the intronic RNA to be bent into a loop or lariate structure. Releasing the intermediate lariate (**Fig 6**) weakens binding to the spliceosome and is important in the context of fidelity of splicing. It permits kinetic competition and determines whether the second esterification proceeds or whether the spliceosome disassembles. Efficient repositioning involves binding of the 3′ splice site and the lariate intermediate to the catalytic core for the second esterification. Failure of the 3′ and 5′ splice sites to become juxtaposed is thought to lead to disassembly of the spliceosome and unspliced transcripts containing splice mutations are subjected to

degradation (**Fig 6**).[92, 104] The U5snRNP has been shown to undergo base pairing to both exons and is believed to align the ends of the two exons for the second esterification to proceed. The two exons join and the ligated product is released from the spliceosome by Prp22, after the second esterification has been completed (**Fig 5**).[105, 106]

1.7.5 Transcription-coupled pre-mRNA splicing

Pre-mRNA splicing occurs either soon after RNA transcription, or co-transcriptionally, during the expression of large or complex genes.[107] The flow of genetic information is tightly coupled and controlled by a highly complex network of functional interactions between the transcription machinery and pre-mRNA processing factors. While usually taking more than 20 minutes *in vitro* (where processing is uncoupled from transcription), post-transcriptional modification *in vivo* can be processed within 30 seconds under circumstances where the transcriptional apparatus and splicing factors are coupled.[108]

The C-terminal domain of RNAP II (CTD), comprising 25 to 52 tandem copies of the consensus repeat heptad $Y_1S_2P_3T_4S_5P_6S_7$,[109] functions as a platform where transcription is coupled and able to interact with the proteins responsible for post-transcriptional modification (viz. capping, splicing and polyadenylation). Mobilisation of the trans-splicing factor to the splice sites and the kinetics of transcription are thought to be the mechanism influencing pre-mRNA splicing.[108] Reversible modifications of the CTD of RNAP II (viz. phosphorylation, glycosylation, and proline isomerisation) provide the basis for what is termed the CTD code. The CTD code refers to the molecular diversity that is introduced to the simple heptad sequence structure by the process of covalent modification (viz. phosphorylation, glycosylation, and proline isomerisation), which gives greater diversity to the processes that regulate transcription initiation and post-transcriptional modification processes. Components of U1snRNPs (viz. U170K, U1A, U1C, TIAR, TIA- 1, and the Sm core proteins) and some other SR proteins, such as U2AF65 and SF2/ASF, have recently been reported to be associated with the RNAP II immunoprecipitate.[110, 111] After the transcription initiation step, proteins associated with RNAP II enhance the capping process and recruitment of other SR proteins (**Fig 7**).[112] These proteins, in turn, stabilise the transcription elongation complex during the process of polymerisation along the DNA template.[113, 114] In 1988, it was hypothesised that the rate of RNA synthesis may affect the secondary structure of

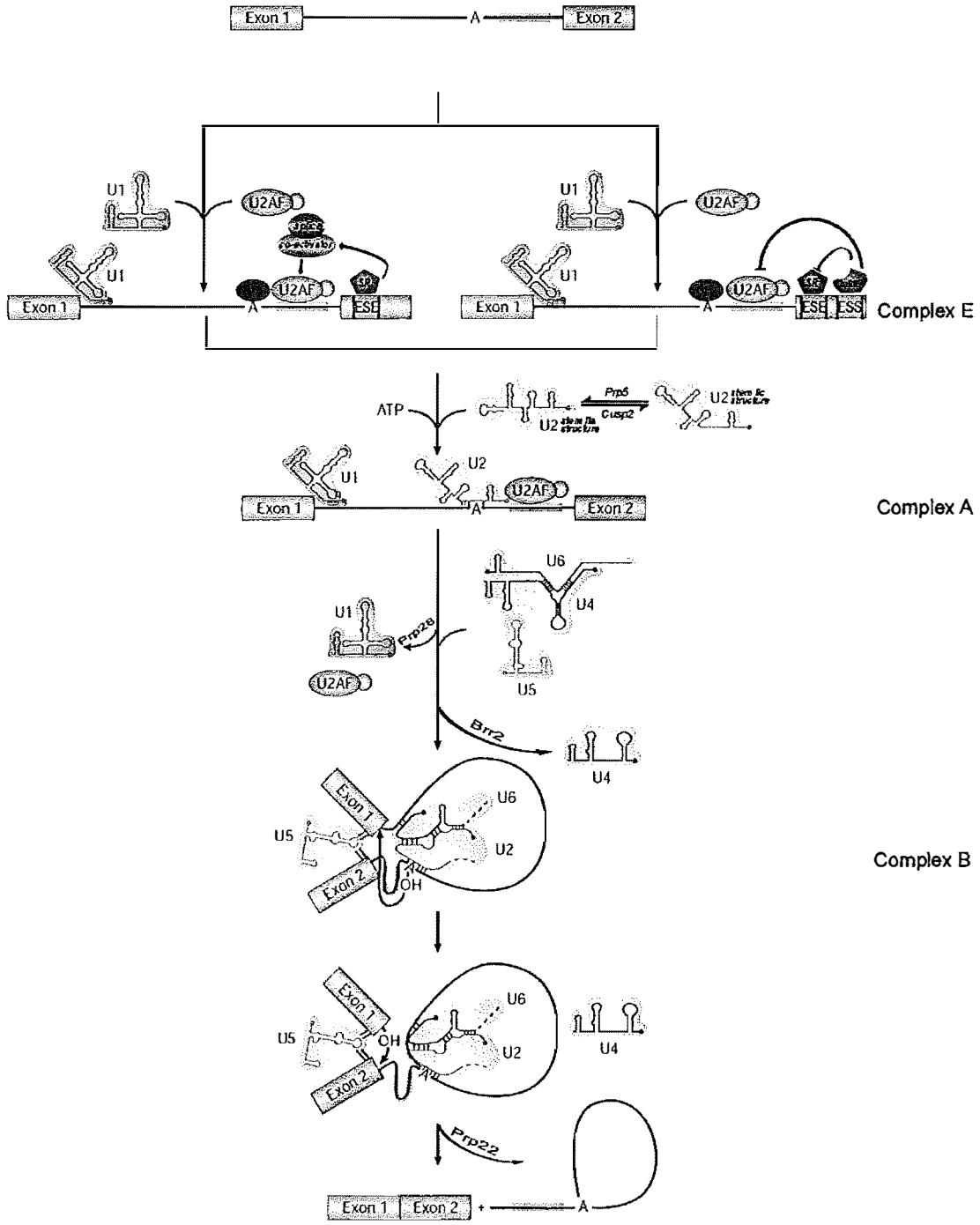

Figure 5: Structure and function of the spliceosome.

The figure illustrates how the spliceosome is thought to carry out the process of pre-mRNA splicing. The complex E contains three essential protein complexes (viz. U1 snRNPs, a branch point binding protein and U2/AF protein). The U1 snRNP complex binds to the 5' splice site via 6-7 base pairing and the branch point binding protein (SF1) becomes located at the branch point. The U2/AF protein recognises a pyrimidine tract. Either serine arginine-rich (SR) proteins or heterogeneous nuclear ribonucleoproteins (hnRNPs) may influence splice site selection through direct interaction with U2/AF or indirect interaction through splicing co-activators. The U2snRNPs attach to a branch site via U2snRNA, which forms a stem IIa structure, thereby forming the A complex The A complex is joined to the U4/5/6 tri-snRNP to form the B complex. In conjunction with rearrangement of U2snRNA from stem IIa to stem IIc through prp5p and cus2p interaction, U6snRNA anneals to a region of U2 snRNA that is near to the branch site duplex bulging 2' hydroxyl group of adenosine. This leads to nucleophilic attack at the 5' splice site, and causes the first esterification reaction. The U5 snRNP undergoes base pairing to both exons and aligns the ends of the two exons for the second esterification reaction to proceed. The two exons join and the ligated product is released from the spliceosome (modified from Patel *et al.* 2003[104]).

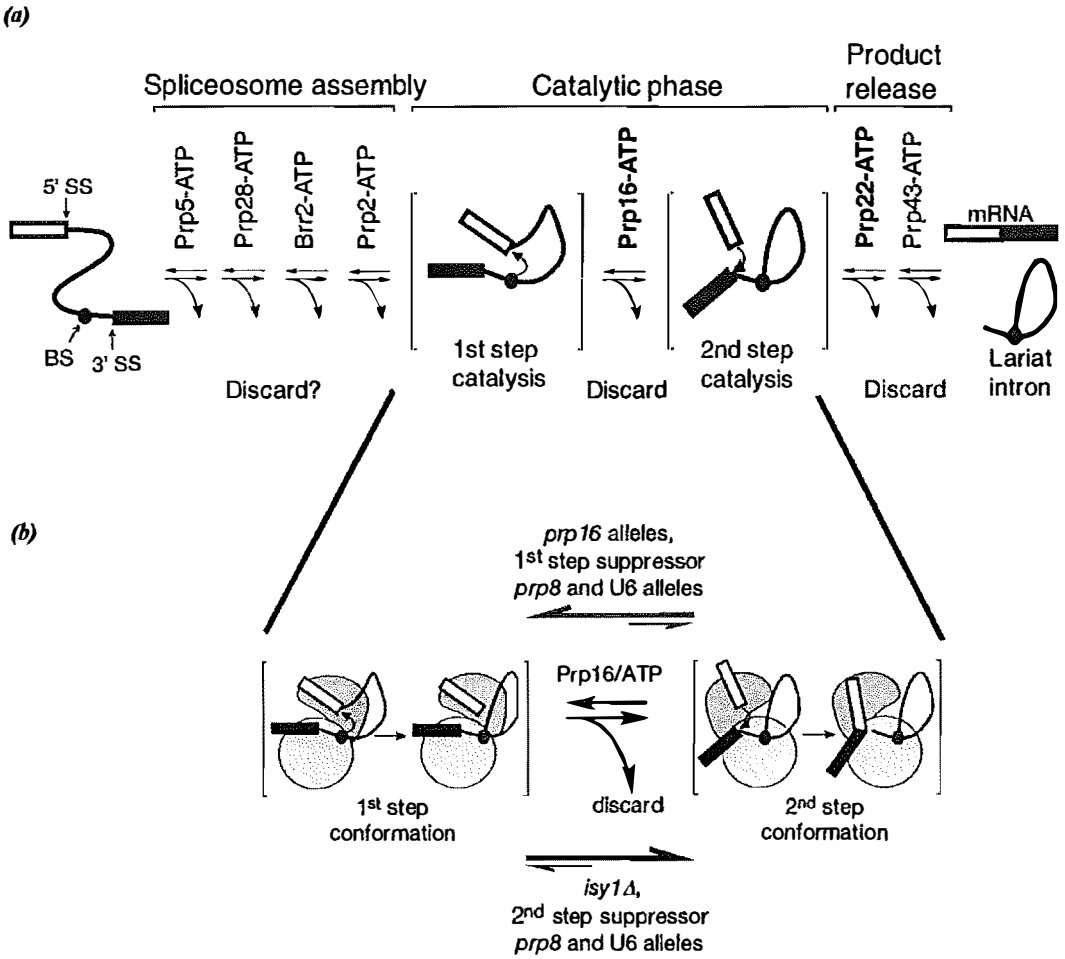

Figure 6: Regulatory mechanism whereby the spliceosome maintains the fidelity of pre-mRNA splicing.

The helicases determine whether pre-mRNA splicing proceeds to completion or aborts through disassembly of the spliceosome complex thereby ensuring the fidelity of pre-mRNA splicing. (a) The process of determining the fidelity of pre-mRNA splicing involves all three of the consecutive stages (viz. spliceosome assembly, the catalytic phase and product release). (b) The conformational alterations in the spliceosome occur after the first esterification reaction to shift the product of the reaction to the catalytic core of the second esterification and this is regulated by interaction between Prp16 and its counterpart, isy1-delta. (modified from Konarska *et al.* 2005[92] and Querry *et al.* 2008[105]).

nascent transcripts, which in turn increase splicing.[116] Evidence supporting this concept came later when the elongation rate of a mutant form of RNAP II, C4, was shown to be increased 3-fold by changing the conformation of the nascent transcript by inclusion of the fibronectin EDI exon in the transcript.[117, 118] Mutated RNAP II was found to partially prevent exon skipping in yeast.[119] The inhibition of specific proteins in the elongation complex; such as topoisomerase I can also affect the splicing process. A reduced number of spliced transcripts and accumulation of splicing factors was reported after treatment with camptothecin, a topoisomerase inhibitor.[111]

1.8 AO-induced exon skipping intervention and choice of chemistry

In 1978, AOs were demonstrated to have the potential for gene downregulation by causing the inhibition of both Rous sarcoma viral translation in a cell-free system and viral replication in chick fibroblast tissue culture.[120, 121] Subsequently, AOs have become of increasing interest to scientists, both as therapeutic agents and as tools to study gene function. During the past two decades, antisense technologies have been continually developed to overcome the practical problems associated with AO usage, including cellular uptake, AO stability (viz. binding capacity of AOs to RNA target), and specificity.[122-124]

The first generation of oligomers, oligodeoxynucleotides on phosphorothioate backbones (ODN), were used to knockdown mature transcripts of various target genes, including the gamma-aminobutyric acid (GABA(A)) receptor,[125] bcl-2,[126] c-myc,[127] transforming growth factor-β (TGF-β),[128] and others. The basic principle of conventional AO downregulation is dependent on annealing of the AO to target mRNA and subsequent RNaseH activation to destroy the message.

The second generation oligomers, 2′-O-methyl modified (2OMe) and 2′-O-methyl and 2′-O-methoxy ethyl (MOE) modified oligomers on phosphorothioate backbones, were devised to minimise nuclease digestion; however 2′ modification, in turn, was shown to compromise RNaseH activation. The concept of using chimeric oligomers (gapmers), in which 8 to 10 nucleotides of DNA are flanked by 5 oligomers of the RNA analogue (2OMeAOs or MOEs), has emerged as a means of maintaining the positive attributes of ODNs (high affinity of AO to RNA molecule and long half-life) without compromising RNaseH activation.

Modification has successfully produced the third generation of oligomers, including phosphorodiamidate morpholino oligomers (PMOs), peptide nucleic acids (PNAs), and locked nucleic acids (LNAs). These third generation oligomers have been used to interfere with the translational process of various gene targets.[129-132] Immediately after the discovery that synthetic double stranded RNA (RNAi) can induce gene inactivation at high potency in mammalian cultures,[133] an explosion of research into therapeutic applications of RNAi has to some extent overshadowed the use of antisense-induced gene inactivation by RNaseH (for review see [134, 135]).

The use of AOs to redirect or modulate the pre-mRNA splicing process is widely recognised as having broad applicability, where second and third generation AOs can be used to sterically mask recognition of splice motifs, and alter the proportions of different products of gene splicing (spliceoforms) without degrading targeted transcripts. Pioneering work on AO-induced splice intervention was initially undertaken in Hela cells, where 2OMeAOs were shown to mask the cryptic splice site of the mutant β-globin artificial gene construct and normalise the splicing pattern.[136] AO-induced exon skipping was first demonstrated to remove human dystrophin exon 19 in Ebstein-Barr virus (EBV) transformed human lymphocytes.[137] Since then, AO-induced exon skipping has been widely studied in relation to the dystrophin gene and provides an example of constitutive splicing manipulation to overcome genetic lesions in both cell culture and animal models.[138-143]

Although possessing greater nuclease resistance, the third generation oligomers, PMOs and PNAs, have low transfection efficiency in cultured cells.[144] To overcome this problem, conjugation with cell penetrating peptides (CPPs) has been used to facilitate oligomer uptake. Systemic administration of peptide-tagged PMOs has led to improved biodistribution and increased exon removal and dystrophin protein restoration *in vivo*.[145, 146] More recently, a carbohydrate-modified peptide-conjugated PMO has been shown to increase dystrophin protein levels by ten to thirty precent in cardiac tissue following systemic treatment in *mdx* mice.[147-149]

1.9 AO-induced exon skipping intervention in the 4^{CV} mouse

In this thesis, the bulk of the work has focused on AO-induced exon skipping in a model of muscular dystrophy, the 4^{CV} mouse, which carries a protein-truncating mutation of dystrophin exon 53. Because removal of both exons 52 and 53 is required

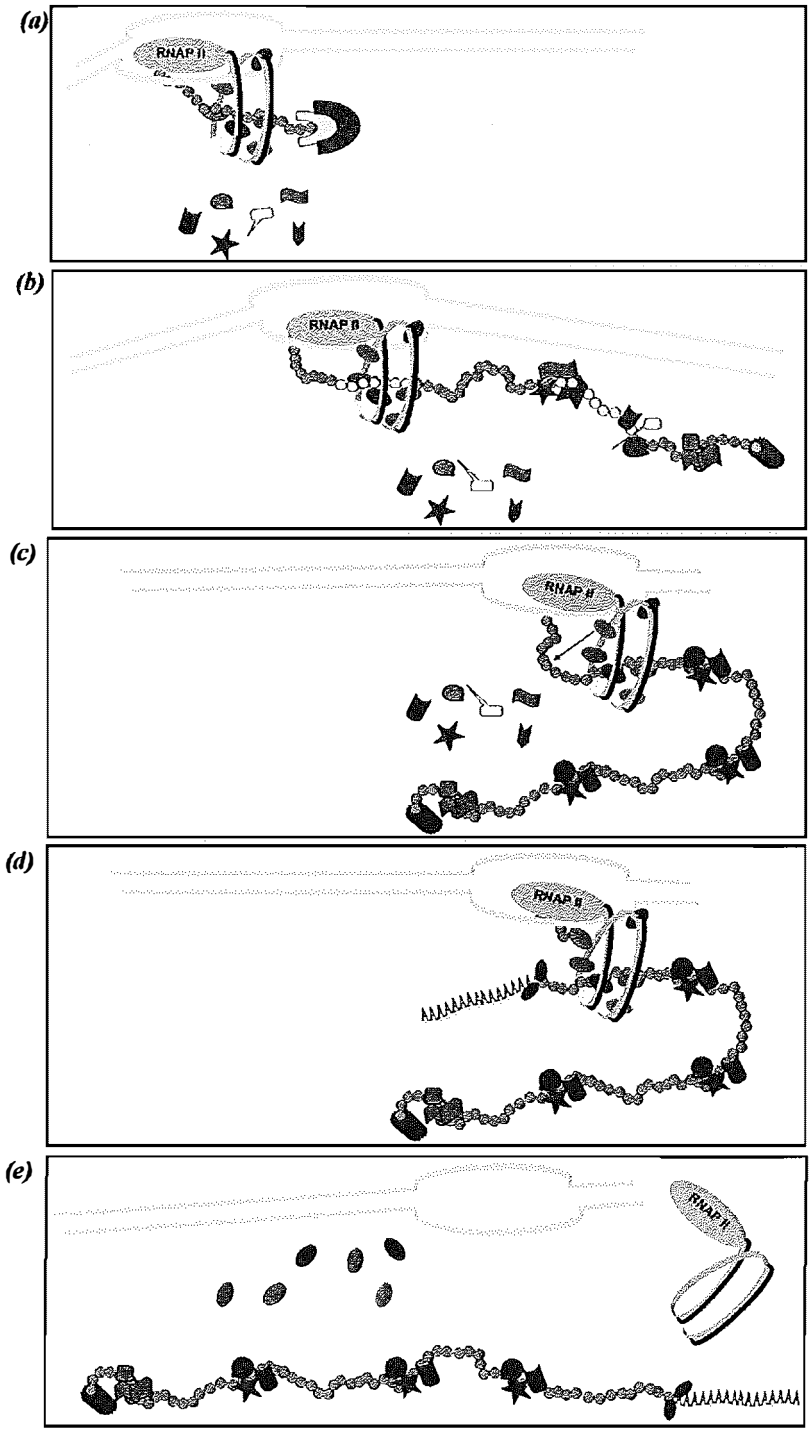

Figure 7: Transcription-coupled pre-mRNA splicing.

The steps in transcription-coupled pre-mRNA splicing are (a) RNA polymerase II (RNAP II) associated with other proteins (ellipsoids attached to the spiral tail of RNAP II) engages the DNA template. As a result, 5' capping enzymes associated with CTD modify the 5' end of the nascent transcript. The 'string of beads' shown represents the mRNA strand containing exonic (light green) and intronic (pale blue) regions; (b) as RNAP II proceeds along the DNA template, proteins associated with CTD mobilise trans-factors to the splice sites, thereby facilitating recognition of splice motifs and initiating pre-mRNA splicing; (c) spliced mRNA is recognised by the exon junction complex (EJC) responsible for mRNA quality control (dark purple proteins); (d) once the RNAP II finishes polymerisation, the proteins associated with the CTD of RNAP II (orange ellipsoid) activate the cleavage of the 3' end of mRNA and recruit the enzyme responsible for polyadenylation. At this stage, the transcript is released and the residual sequence still attached to RNAP II is degraded by 5' to 3' exonuclease to promote RNAP II dissociation from the DNA; and finally (e) the complex disassembles allowing the mature transcript to be transported to the nuclear pore.

to maintain the reading frame, the 4^{CV} model offers an opportunity to investigate dual exon skipping in a region that corresponds to the major mutation hot spot in the human dystrophin gene.

1.10 AO-induced exon inclusion in SMN2 gene transcript

Although the major focus of the research described here involves exon removal in the 4^{CV} mouse model of muscular dystrophy, the thesis also reports attempts to restore gene expression by the inclusion of selected exons into other mature transcripts. These studies were undertaken on the premise that it may be possible to affect change by masking silencing elements or by adding AOs which reinforce exon selection and inclusion. By comparison with exon skipping, only limited numbers of AO-induced exon inclusion experiments are reported in the literature, although this is now starting to increase. The survival motor neuron gene is one of the best characterised models.[150-152]

Spinal muscular atrophy (SMA) is characterised by degeneration of the anterior horn cells leading to four main symptoms, hypotonia, symmetrical muscle weakness and atrophy, tremor of finger and hand, and fasciculation of the tongue muscles. A defect in the survivor motor neuron gene (SMN) has been identified as the cause of SMA. In humans, there are two copies of the SMN gene, SMN1 (telomeric SMN) and SMN 2 (centromeric SMN). SMN2 differs from SMN1 by one nucleotide in the splice motif of exon 7, which leads to production of a transcript lacking exon 7, while the full length protein is almost exclusively produced by SMN1. SMN1 is missing or altered in SMA patients, but SMN2, which mainly encodes a truncated protein, can also produce limited amounts of the full length transcript.

One of the most promising genetic treatments for this disease is enhancement of the number of transcripts including exon 7, where modified antisense oligomers have been shown to reinforce exon inclusion in human cultured fibroblasts.[153-155] Studies reported in this thesis aim at identifying potential sites for AO binding as a new avenue for AO-induced exon inclusion therapy for SMN patients, a technique that may have broader applicability to other diseases.

1.11 The goals of the thesis and their significance

DMD is a relentlessly progressive neuromuscular disease which affects one in 3,500 newborn males. Because, to date, no effective treatment for DMD is clinically

available, patients usually lose ambulation and die from respiratory and cardiac complications in their late twenties. The use of antisense oligomer-induced exon skipping has already been the subject of clinical trials in DMD patients.[156, 157] The novelty of the works presented here is that it explores the concept of personalised therapy for DMD, whereby oligomers are constructed to specifically target individual mutations. These studies use a proven animal model of DMD, the 4^{CV} mouse, which carries a protein-truncating mutation of exon 53. Importantly, exon 53 corresponds to a mutation hot-spot in human dystrophin (exons 45-53), thereby making the 4^{CV} mouse a particularly relevant model of DMD in humans. The model also offers an opportunity to investigate dual exon skipping, which in theory, has the potential to extend the range of mutations that may be treatable by use of antisense oligomers.

For personalised oligomer therapy to be effective in individual DMD patients, a method to optimise the therapeutic oligomers in appropriate cultured cells is required. The thesis explores the use of cultured human myoblasts, from both unaffected humans and a DMD patient with a mutation in exon 16, as a means of establishing the most effective oligomers to induce exon 16 skipping in a range of patients with mutations that are treatable by removal of exon 16.

The thesis also investigates the use of antisense oligomers to induce exon inclusion in SMA patients using cultured patient fibroblasts, with the goal of exploring the applicability of antisense oligomers in the treatment of other neuromuscular disorders.

1.12 Purpose

The aims of this project are to explore the following issues:

1. How efficiently can AOs modify splicing patterns?
2. Can dual exon skipping be efficiently induced in the B6Ros.Cg-*Dmd*$^{mdx-4Cv}$/J mouse model of muscular dystrophy?
3. Does the choice of oligomer chemistry influence exon skipping efficiency *in vivo*?
4. Do combinations of AOs that induce exon removal lead to increased dystrophin levels and improve muscle functionality?

5. Can the use of oligomers for exon removal be directly extrapolated between species?

6. Does the use of AO-induced exon skipping in normal human myoblasts provide a means of optimising the AOs to be used clinically in DMD patients?

7. Can appropriately designed AOs promote exon inclusion in fibroblast cultures from an SMA patient and, if so, what combination of oligomers is most optimal?

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CHAPTER 2

Personalised genetic intervention for Duchenne muscular dystrophy: Antisense oligomers and exon skipping

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2.1 Abstract

Duchenne muscular dystrophy (DMD) arises from protein-truncating mutations in the large dystrophin gene that preclude synthesis of a functional protein that primarily stabilizes muscle fibre membranes. The absence of dystrophin leads to this most common and serious form of childhood muscle-wasting. Since the identification of the dystrophin gene in 1987, cell and gene repair or replacement therapies have been evaluated for DMD treatment and one genetic intervention, exon skipping, is now in clinical trials. Antisense oligomers have been designed to redirect dystrophin splicing patterns so that targeted exons may be removed from a defective dystrophin pre-mRNA to either restore the reading frame of a deletion, or excise an in-frame exon corrupted by a nonsense mutation or micro-insertion/deletion. This review discusses the evolution of oligomer induced exon skipping, including *in vitro* applications, evaluation of different oligomer chemistries, the treatment of animal models and alternative exon skipping strategies involving viral expression cassettes and ex vivo manipulation of stem cells. The discussion culminates with the current clinical trials and the great challenges that lie ahead. The major obstacle to the implementation of personalised genetic treatments to address the many different mutations that can lead to DMD, are considered to be establishing effective delivery regimens for the different patients and their mutations. Furthermore, the view of regulatory authorities in assessing preclinical data on potentially scores of different but class-specific compounds will be of paramount importance in expediting the clinical application of exon skipping therapy for this serious and relentlessly progressive muscle wasting disease.

Keywords: Antisense Oligonucleotides, Exon skipping, Duchenne muscular dystrophy, Morpholino, clinical trials, dystrophin, personalized medicine, pre-mRNA splicing

Abbreviations: AO: antisense oligomer, BMD: Becker muscular dystrophy, cDNA: complementary DNA, DMD: Duchenne muscular dystrophy, DNA: deoxyribonucleic acid, ENAs: 4′-C-ethylene bridge nucleic acids, ESE: exon splicing enhancer, GRMD: Golden retriever muscular dystrophy, kDa: kilodalton, LNAs: locked nucleic acids, MOE: 2′-O-methoxy-ethoxy AO, NMD: nonsense mediated decay, ODN: oligodeoxynucleotide, PMO: Phosphorodiamidate Morpholino Oligomer, PNAs: peptide nucleic acids, pre-mRNA: precursor messenger ribonucleic acid, RNA:

ribonucleic acid, SCID: severe combined immunodeficiency, 2OMe: 2'-O-methyl modified.

2.2 Introduction

When considered individually, serious monogenic disorders are fortunately relatively rare, especially when compared to complex traits such as Alzheimer's, asthma, diabetes, or acquired conditions, including pathogenic infections and cancer. The most common human autosomal recessive disorder, cystic fibrosis, is reported to occur at a frequency of about 1 in 2100 in the Caucasian population.[1] However, when viewed collectively, single gene disorders are an enormous burden to those affected, their families, communities, and the health care system.

It is no longer appropriate to consider single gene disorders as "simple", particularly since it is apparent that different lesions in a particular gene can result in a variety of clinically distinguishable conditions, with either recessive or dominant modes of transmission. Although the ΔF508 mutation defect accounts for about 75% of cystic fibrosis cases,[2, 3] over 1,000 different mutations have been reported and the clinical presentation can vary considerably from severe, with extensive lung involvement, to a very mild phenotype with reduced fertility as the predominant symptom. Similarly, different mutations in the huge dystrophin gene can lead to the allelic conditions, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), and X-linked dilated cardiomyopathy.[4]

Restoration of expression of a single component to address a missing or defective gene product is the basis of gene replacement (viral and non-viral systems) or cell-based therapies. Although great advances are being made in the treatment of some conditions, for example, gene replacement in children with X-linked severe combined immune deficiency (SCID),[5, 6] there have also been serious adverse events and progress has not been as rapid as anticipated.[7] Challenges to gene and cell replacement therapy for DMD arise from the size of the gene product and/or nature of the gene expression. Dystrophin, the affected gene product in DMD, is encoded by the largest known gene and is expressed as multiple isoforms in different tissues, with the 427 kDa skeletal muscle protein produced at low amounts in about 30% of the human body mass.[8]

Such challenges in gene and cell replacement for DMD have prompted the examination of other possible therapeutic interventions, including up-regulation of a homologous protein or redirecting expression, processing, or translation of the defective gene product.[9-11] This review will consider the use of antisense oligomers (AOs) to

reduce the severity of DMD, through specific redirection of dystrophin pre-mRNA processing to by-pass protein-truncating mutations during mRNA maturation. The nature of this genetic therapy is such that specific AOs and exon skipping strategies must be tailored to address different mutations. Although this may sound daunting, development of personalized treatments should not be considered unrealistic. Many compounds have now been optimised to address different dystrophin mutations *in vitro*, and two compounds are currently in clinical testing to restore the reading frame of a common type of dystrophin mutation. Demonstration of dystrophin expression in these trials should facilitate implementation of other AO strategies for different DMD patients, particularly if exon skipping could be regarded as a generic therapy.

2.3 Duchenne and Becker Muscular Dystrophy

DMD and BMD are allelic X-linked recessive conditions arising from mutations in the dystrophin gene. One third of cases are *de novo* and germ-line mosaicism has been reported to be as high as 14%.[12] The tremendous size of the dystrophin gene and organization of repeated elements within some introns in excess of 200kB, are thought to contribute to the high spontaneous mutation rate. DMD occurs at a cited incidence of 1 in 3,500 live male births, and is the most common severe muscular disorder in childhood.[13] DMD individuals appear normal at birth, but present with muscle weakness between the ages of 3-5 years. Muscle degeneration gradually overwhelms regenerative capacity in a relentlessly progressive and predictable manner. As muscle degeneration progresses, affected individuals exhibit difficulties arising from the floor, climbing stairs and running, and eventually lose ambulation before the age of 12 years. The most common causes of death are cardiac or respiratory complications, but improvements in heath care, the use of steroids and assisted ventilation have extended the life span of DMD patients by up to 50% over the last two decades.[14]

BMD is estimated to occur at one tenth the frequency of DMD.[15] Presenting with a spectrum of severities, BMD is clinically classified as an individual becoming wheelchair bound by age 16 years or later, while some are apparently asymptomatic and may only be diagnosed later in life.[16] Examples of gross dystrophin gene deletions that have been identified in mildly affected BMD patients are shown in **Table 1**, where it can be seen that over 50 dystrophin exons may be deleted, in various combinations, with relatively minor clinical consequences. It seems probable that the low incidence of

BMD, compared to DMD, may be due to the inability to identify cases that do not present with overt symptoms. A mutation in the dystrophin gene was recently reported where the loss of exon 16 did not raise the serum creatine kinase levels,[17] a sensitive marker of muscle damage.

The molecular distinction between DMD and BMD depends upon the quantity and quality of dystrophin that can be synthesized as a consequence of the gene lesion.[18] Genomic deletions of one or more exons, found to cluster in 2 hotspots near huge introns, are the most common type of dystrophin mutation and account for about 60% of cases. Duplications, typically involving multiple exons, are generally found in the proximal third of the gene and are seen in 8-15% of DMD cases.[19-22] Disruption of the reading frame that leads to premature termination of dystrophin synthesis results in DMD, while in-frame gene re-arrangements typically allow the generation of internally truncated dystrophin that retains some biological function. The variable manifestation of BMD, from border-line DMD to asymptomatic, reflects the quality and/or quantity of the internally shortened dystrophin (for review see [23-25]).

The dystrophin gene contains 79 exons spanning approximately 2.4 million base pairs and encodes 3 major isoforms, which are expressed primarily in muscle, heart and brain. The reading frame organization of the 79 exons is shown in **Figure 1**. In addition, there are 4 internal promoters, which encode shorter isoforms expressed in other tissues. Due to the catastrophic consequences of dystrophin loss on muscle function and integrity, it is presumed that the 427 kDa skeletal muscle isoform is most important, hence this has been the focus of the gene repair or replacement studies (for review see [26, 27]).

Dystrophin is thought to act much like a shock absorber linking the actin cytoskeleton to a complex of proteins embedded in the sarcolemma. The primary actin binding domain involves dystrophin exons 2-8, although secondary actin binding sites have been identified,[28, 29] supported by the identification of mildly affected BMD individuals missing exons 3-9.[16] Dystrophin exons 62-69 encode the cysteine rich domain, which is pivotal in the linkage of dystrophin to β-dystroglycan, and hence to the dystrophin associated proteins and glycoproteins embedded in the sarcolemma. In addition to the primary structural role linking the actin cytoskeleton and the sarcolemma, dystrophin also anchors syntrophin, dystrobrevin, and localizes nNos.[25] As well as the major 427 kDa isoforms expressed in muscle, heart and brain, internal

promoters located within introns 30, 45, 56 and 63 are responsible for the production of the shorter isoforms, DP260, DP116, DP140, and DP71, respectively. In non-muscle tissue, DP116 associates with the sarcoglycan complex and is implicated in myelin stability in schwann cells.[30] Transient expression of DP140 in embryonic kidney is involved in renal tubulogenesis.[31] The carboxy (C)-terminal domain of DP71 isoforms plays an important role in neuronal differentiation.[32]

The actin and β-dystroglycan binding domains are separated by 24 spectrin-like repeats, and this rod domain is encoded by about two thirds of the dystrophin gene transcript.[33] Portions of the rod domain appear to be somewhat redundant in that substantial in-frame deletions of rod domain generally lead only to a mild BMD phenotype (**Table 1**).[34]

2.4 Natural precedents for exon exclusion during dystrophin processing

There are two precedents to support the hypothesis that induced exon skipping could minimise the consequences of protein-truncating mutations in the dystrophin gene. Revertant fibres are dystrophin-positive fibres that occur naturally and have been detected in *mdx* mouse model of muscular dystrophy,[35, 36] the dystrophin-deficient muscular dystrophy canine model,[37-39] and at least 60% of all DMD cases.[40-43] Another canine model, a German short-haired pointer,[44] in which the entire dystrophin gene was missing, did not contain any revertant fibres, suggesting that the mechanism responsible for restoring some dystrophin expression required the retention of sufficient coding sequences of the dystrophin gene.

Although revertant fibres are regularly detected in many dystrophic tissues, the frequency is insufficient to be of any substantial clinical benefit.[42] RNA analysis and epitope mapping indicated the presence of multiple revertant dystrophin isoforms in human and mouse muscle. This suggested some natural exon skipping event led to re-arrangements,[36, 45] generally excluding 20 or more exons.[46] *In situ* hybridization studies in the *mdx* mouse using exon 23 and flanking intronic sequences as a genomic probe, indicated that the dystrophin gene in the majority of revertant fibres was structurally intact, thereby excluding secondary somatic deletions in the dystrophin gene as the primary possible mechanism.[46] The underlying basis of revertant fibres is believed to involve some form of alternative splicing,[36, 47] although it is difficult to conceptualize a

mechanism whereby 20 exons spanning hundreds of kilobases of pre-mRNA are consistently excluded in one muscle fibre and not another.

BMD patients typically have genomic deletions in the dystrophin gene that do not disrupt the reading frame. A few point mutations that lead to BMD have been identified, including missense mutations in the actin binding domain,[15] and splice motif mutations that either lead to excision of an in-frame exon, or reduce the amount of normal dystrophin mRNA.[48] Some nonsense mutations in the dystrophin gene have also been found to lead to BMD,[49, 50] in apparent conflict with the reading frame hypothesis, which predicts that protein-truncating mutations should result in DMD.[51] However in these milder than expected BMD cases, it was found that the nonsense mutation influenced splicing patterns such that natural exon skipping excluded the exon containing the nonsense mutations.[50] Comprehensive screening of genomic DNA has been reported to confirm diagnosis of over 96% of DMD cases, whereas only 60% of BMD mutations were identified.[52, 53] Analysis and sequencing of dystrophin cDNA from muscle is often essential to identify the consequences of less obvious gene mutations, where a nonsense or missense mutation/polymorphism may lead to exon skipping or activation of a cryptic splice site. Similarly, deep intronic DNA changes, sometimes kilobases away from the nearest dystrophin exon, can lead to the incorporation of pseudo-exons in the mature gene transcript.[54]

2.5 Antisense Oligomers and Modes of Action

An oligodeoxyribonucleotide was reported to inhibit Rous sarcoma virus replication in cell culture nearly three decades ago.[55, 56] Antisense technologies became synonymous with gene down-regulation studies, most commonly through the induction of RNaseH degradation of the RNA strand in the AO:RNA duplex. Inadequate controls confounded results of some early experiments, where apparent gene suppression arose from non-sequence specific effects, in particular the sequestration of transcription/translation proteins by the phosphorothioate backbone. Despite these early setbacks, which saw antisense technology regarded with a great deal of skepticism, there has since been great progress in terms of new chemistry development and identifying distinct mechanisms of altering gene expression.

New oligomer chemistries, with modified bases and/or backbones, generated compounds with greatly increased annealing affinity and/or enhanced resistance to

nuclease degradation. Vitravene (fomivirsen) was the first antisense drug to achieve marketing clearance in the USA to treat cytomegalovirus retinitis in people with AIDS.[57] In addition, new oligomer chemistries were able to influence gene expression through mechanisms other than RNaseH-induced degradation. Chemically synthesised RNA oligomers are routinely used in gene silencing studies in nematodes, plants and human cells.[58-61] Some second or third generation oligomers, which were not able to activate silencing pathways, can modify gene expression at different stages. Depending upon the target sequence design, some modified oligomers can suppress translation by masking motifs essential for ribosomal initiation and elongation, while other oligomers can anneal to motifs involved in exon recognition and intron removal, thereby redirecting pre-mRNA processing.

2.6 Early splice intervention studies

Kole and colleagues first reported the use of AOs to correct aberrant β-globin pre-mRNA splicing fifteen years ago.[62] Intronic mutations in this gene activated cryptic splice sites and account for nearly 30% of β-thalassemia cases. Despite the presence of intact splice donor and acceptor sites, the selection of cryptic splice sites led to some intron retention in the mutant β-globin mRNA. AOs consisting of 2'-O-methyl modified bases on a phosphorothioate backbone (2OMe) were designed to anneal to the cryptic splice sites, rendering them inaccessible to the splicing machinery, which in turn defaulted to the selection of the normal splice sites. A few years later, Matsuo and colleagues were able to induce an abnormal dystrophin gene transcript by targeting an oligodeoxynucleotide on a phosphorothioate backbone (ODN) to an internal domain within dystrophin exon 19.[63] The "Kobe" DMD mutation was found to arise from a 52 bp deletion within dystrophin exon 19 that, while leaving the donor and acceptor sites intact, resulted in the excision of the entire exon from the gene transcript.[64, 65] Matsuo and colleagues hypothesized that the small intra-exonic deletion removed crucial exon recognition motifs and resulted in complete exon removal. An AO was designed to induce dystrophin exon 19 skipping in normal lymphoblastoid cells transformed with Epstein-Barr virus.[63] Hence, the first demonstration of exon skipping in the dystrophin gene did not rescue defective gene expression, but induced abnormal splicing and disrupted the reading frame. Nevertheless these experiments confirmed the principle of targeted exon skipping and proposed this approach as a potential therapy for DMD.

2.7 Animal Models

The most commonly studied animal models of dystrophin mutations are the *mdx* mouse, which carries a nonsense mutation in exon 23,[35] and the Golden retriever muscular dystrophy model (GRMD), which was found to have a splice site mutation that leads to exon 7 exclusion and a subsequent mRNA frame-shift.[39] The *mdx* mouse model is readily available, inexpensive, but suffers from the limitation that the dystrophin deficiency does not induce an overt severe dystrophic phenotype. Nevertheless, detailed examinations show raised serum creatine kinase levels and muscle weakness, and some muscles, in particular the diaphragm, show extensive fibrosis and dystrophic pathology.[66] As these animals age, the consequences of the dystrophinopathy become more obvious, but these animals can voluntarily run in a manner similar to wild type animals. Another mouse model, which does show a very severe phenotype is the utrophin/dystrophin double knockout mouse,[67] although no exon skipping studies in this model have been reported to date.

The canine GRMD model is much more severely affected, perhaps more so than the human condition, and provides a more relevant model in which to assess any therapies. Limitations of the canine model include substantial variation in severity, excessive costs involved in maintenance and care, limited numbers available from each litter, and the emotive issues associated with working on companion animals.

2.8 AO Design and Target Selection

The rescue of dystrophin expression using AO-induced exon skipping in the *mdx* mouse was first reported by Dunkley and colleagues, who described a 2OMe AO 12mer directed at the exon 23 acceptor splice site that generated a transcript in which exons 23 to 29 had been skipped. 2OMe AOs directed at the branch point or the donor splice site of exon 23 were not found to have any effect on the dystrophin mRNA processing.[68] In contrast, the following year we demonstrated that targeting the exon 23 donor splice site with a 2OMe AO 20mer was able to induce precise and consistent exon 23 skipping, whereas a 20mer directed at the acceptor site was found to be ineffective.[69] Subsequent refinements in AOs targeting the dystrophin exon 23 donor splice site were reported and found to further enhance the capacity of the AO to induce exon 23 skipping.[70] Additional AO refinement at the acceptor splice site consistently failed to induce any detectable skipping.[70]

Studies describing the application of AOs directed to exons flanking frame-shifting genomic deletions in DMD patient cell lines were reported by van Deutekom and colleagues.[71] The Leiden muscular dystrophy database (http://www.dmd.nl/) lists exon 45 as one of the most commonly deleted exons in DMD, whereas the in-frame deletion of exons 45 and 46 is associated with a mild form of BMD. van Deutekom and colleagues designed a 2OMe AO to motifs within exon 46 and conclusively demonstrated restoration of the reading frame in cells from DMD individuals carrying a dystrophin genomic deletion of exon 45.[71] These studies were then extended to a variety of other mutations in the dystrophin gene.[72-74]

It has been proposed that only 12 different AOs would be able to restore the reading frame in the majority of DMD deletion patients, particularly since this type of mutation is clustered in the minor and major dystrophin deletion hotspots.[75] Indeed, the most commonly deleted exons lie between exons 45 and 55, and Beroud and colleagues,[76] reported that multiple exon skipping across those exons could restore some functional dystrophin expression in almost two thirds of all DMD patients.

However, more than one-third of DMD cases do not arise from genomic deletions and these patients should not be excluded from any potential exon skipping therapy. Nonsense mutations, splicing defects and micro-insertion/deletions have the potential to lead to premature termination of translation, and these defects appear evenly distributed across the entire gene. As discussed previously, an apparent catastrophic DNA change such as a nonsense mutation, does not necessarily lead to premature termination of translation, if the DNA variant compromises exon recognition and results in variable levels of natural exon skipping. Since the excluded exon is in-frame, a protein typical of BMD is generated,[49, 50] again providing evidence that exon skipping has the potential to ameliorate DMD progression.

Furthermore, unlike many genomic deletions involving multiple exons, the entire dystrophin coding region is present in the non-deletion DMD patients. The removal of one or two exons to by-pass a protein-truncating mutation is unlikely to seriously compromise the function of the induced dystrophin, unless the exons code for a crucial functional domain. Aartsma-Rus and colleagues,[77] described 114 AOs that target 35 exons for removal. In 2007, we released the first draft of AOs targeting every exon in the dystrophin pre-mRNA for excision, excluding the first and last exons.[78]

It has been suggested that directing AOs to dystrophin donor or acceptor splice sites may lead to off-target effects on other splice sites,[79] a possibility which cannot be discounted. However, the invariant bases of the acceptor and donor splice sites are only 2 nucleotides long, and occur at the end and beginning of each intron flanking the target exon (---ag[EXON]gu---). We took the approach that any motif involved in splicing must be regarded as a potentially amenable target and evaluated the efficiency of AOs directed at acceptor and donor splice sites, as well as Exon Splicing Enhancers (ESE's) as predicted by ESEFinder.[80, 81] Although the "ag" and "gu" motifs are almost invariant at the acceptor and donor splice sites respectively, these two nucleotides would only constitute a minor proportion of the AO annealing site. It may be argued that targeting ESE's, where 6 or 8 consensus nucleotide motifs are recognised by SRp55 or SC35 respectively, offers a greater chance of cross-transcript targeting.

Remarkably from these two extensive reports on AO design to induce dystrophin exon skipping,[77, 78] about two thirds of AOs designed and evaluated were able to induce some level of targeted exon exclusion. This is consistent with the observation that many changes in the protein coding region can disrupt splicing,[82] and implies that many motifs are involved in exon recognition and splicing. However, there are substantial variations in exon skipping efficiencies, as clearly some AOs targeted more amenable or responsive sites for induced exon skipping than others. Some exons were readily and efficiently removed at what was arbitrarily chosen an acceptable level *in vitro*, greater than 30% exon removal compared to the intact transcript after transfection at 100 nM AO:lipoplex,[78] while other exons were more difficult to dislodge. Aartsma-Rus and colleagues,[83] noted that the effective AOs targeted significantly higher numbers of SF2/ASF, SC35 and SRp40 motifs than the ineffective AOs.[77]

Our strategy for AO design has been more empirically based. An initial panel of AOs was designed to target splice site junctions for each exon, as well as predicted ESE motifs. Normal myogenic cultures were transfected and the test compounds exhibiting the most pronounced exon skipping efficiency were used as a template to design a subsequent series of overlapping AOs. The most efficient AO was defined as the compound that induced maximal exon excision *in vitro*, after transfection at concentrations over the range 10 to 100 nM. Although the AOs designed to induce specific exon skipping will ultimately be applied to cells expressing a defective

dystrophin gene, AO development was undertaken in normal human primary myogenic cell cultures. Designing AOs to target the normal dystrophin gene transcript places extra demands on evaluation. Unlike cells expressing a defective dystrophin mRNA subjected to increased turn-over through nonsense mediated decay,[84] removal of approximately half of the exons from a normal dystrophin transcript may lead to a disruption of the reading frame. Hence, the normal gene transcript will be expressed at wild-type levels, and the induced exon deleted transcript will be subjected to faster turn-over though nonsense mediated decay. Consequently, when the appropriate therapeutic oligomer is applied to dystrophic cells, the effect on exon skipping should be more pronounced, as the reading frame will have been restored and the induced transcript no longer subjected to nonsense mediated decay (NMD). This feature was evident in evaluating AOs to excise exon 19 from the dystrophin gene transcript expressed in normal and *mdx* murine myogenic cells. The *mdx* dystrophin transcript would be subjected to NMD, as would any dystrophin transcripts missing exon 19. Despite the same nucleotide sequence and splicing machinery, exon 19 removal was induced in *mdx* cells at concentrations 4 fold lower than that required in the normal cells.[85]

One trend in AO design that became evident was that the length of the AO could play a major role in determining the efficacy of induced exon skipping, although this appears to be largely dependent upon the target exon.[86] Several motifs were examined as targets for induced skipping of human dystrophin exon 16, in particular, a cluster of high-scoring potential ESE's near the donor splice site. Despite masking of high-scoring ESE's and this donor splice motif, overlapping AOs directed at the human exon 16 donor site were found to be ineffective, whereas a 25mer, spanning the acceptor site induced moderate exon 16 skipping. AOs with additional bases at the 5' or 3' end of the 25mer were found to be about four-fold more effective than the original AO targeting the acceptor. Most surprisingly, a 20mer common to all three AOs was found to be totally ineffective.[86] Although it had been observed that a 25mer directed at the mouse exon 23 donor site was marginally more effective than a 20mer, longer AOs (30mer) directed at this site were consistently found to induce less exon 23 skipping than the shorter compounds.[86]

Some dystrophin exons were difficult to dislodge from the mature dystrophin mRNA, and despite designing AOs across most of the exon, with either no or only very low levels of skipping being induced after transfection with high AO:lipoplex

concentrations. Exon 20 was one such example, in which over 20 different AOs were designed and evaluated, but only one compound was eventually found to induce moderate levels of exon 20 excision. Combinations of AOs were then evaluated and some, but not all AO cocktails, were found to be very effective in a clearly synergistic rather than cumulative manner.[83, 87] AO cocktail design was not as simple as combining the most effective AOs, as the optimal AO cocktail for exon 20 consisted of two compounds that had no effect on splicing when used individually. Replacing one of the AOs in this cocktail with a longer overlapping compound that did show some exon skipping potential when used alone, actually lowered the efficiency of that cocktail.[87] In another example, very weak exon 65 skipping could be induced with one AO after transfection at a concentration of 600 nM. When this AO was combined with another directed at exon 65, pronounced exon skipping was evident after transfection at a combined AO concentration of 2 nM.[87]

This raises the question of exactly how AOs influence the splicing process. It had been assumed that AOs anneal to single stranded motifs on the pre-mRNA, where SR proteins or other splice factors, such as short non-coding RNAs including miRNAs [88] may be involved in exon recognition and definition. AO binding to the appropriate target would render that pre-mRNA site double-stranded and presumably prevent correct assembly of the spliceosome. If this were the case, one would assume that the more obvious motifs involved in splicing, such as the acceptor or donor sites should provide reliable targets for consistent splice intervention. This is clearly not the case, as we have identified only one human dystrophin exon in which the donor splice site was the single most amenable target for exon skipping.[78] Although directing AOs to some donor splice sites does induce exon skipping, there are more examples of no skipping whatsoever. As reported by Arechavala-Gomeza *et al,*[89] applying a panel of AOs to micro-walk across the donor splice site of human dystrophin exon 51, or using AOs of increased length targeting the donor site, failed to induce any substantial exon skipping. It would appear that if a donor splice motif does not appear amenable, extensive AO design and manipulation targeting that area will be a futile exercise.

Rather than directly masking motifs recognized by the various splice factors, perhaps the AOs bind to the pre-mRNA and alter secondary structures that are crucial in exon recognition and splicing. There is mounting evidence that secondary structures within the pre-mRNA are involved in both constitutive and alternative splicing,[90-92] and

this may account for the observations that 2 out of 3 AOs designed and evaluated were able to induce some exon skipping.[77, 78] It is possible that some donor or acceptor sites unresponsive to AO intervention are influenced by particular splicing factors, which bind very strongly and/or immediately after transcription.

Several oligomer chemistries have been identified as suitable to induce exon skipping. While ODNs were first used to induce exon 19 excision in dystrophin processing,[93] and later used in the first clinical trial involving one patient,[94] there are several reasons why this particular chemistry should not be taken to the clinic for induced exon skipping. This type of oligomer is more susceptible to nuclease degradation than many other chemistries and would need constant re-administration to maintain therapeutic concentration. Secondly, ODNs are typically used to induce degradation of the target gene transcript by RNaseH action. Presumably if exon excision can occur before RNaseH degradation, the induced transcripts would then be resistant to degradation, unlike the intact transcript. Although ODNs were able to induce exon 19 skipping in cultured cells, AOs of this chemistry directed at other splice motifs did not induce exon skipping.[85] Chimeric AOs consisting of a mixture of modified and unmodified bases demonstrated increased exon skipping efficiency, correlated with increased content of 2'-O-methyl modified bases.[95]

One of the more commonly used nucleotide chemistry for AOs to induce exon skipping are those consisting of 2'-O-methyl modified bases on a phosphorothioate backbone.[62, 69, 71, 96] Several other AO chemistries have also been evaluated for induction of exon skipping, including terminally modified 2OMe AOs,[97] 2'-O-methoxy-ethoxy AOs (MOE, unpublished data), 4'-C-ethylene bridge nucleic acids (ENAs),[72, 95, 98] locked nucleic acids (LNAs),[72, 99] peptide nucleic acids (PNAs),[72] and phosphorodiamidate morpholino oligomers (PMOs).[72, 100] LNA, PNA, PMO, and 2OMe AOs were directly compared to remove exon 46 from a DMD cell line in which exon 45 was deleted.[72] This study found that oligomers prepared as 2OMe and LNAs could efficiently induce exon 46 removal, whereas the equivalent compounds prepared as PMOs or PNAs were ineffective.[72] These authors concluded that the 2OMe chemistry was preferable to pursue further induced exon skipping strategies,[72] and an AO of this chemistry has now undergone Phase I clinical trials.[101, 102]

There are advantages and disadvantages to each of the AO chemistries, and several factors must be taken into account. The LNA compound designed to excise

exon 46 was able to induce substantial exon skipping, has the additional advantages of increased resistance to nuclease degradation and an exceptional affinity for the target sequence. A 15mer targeting exon 46 was estimated to have a Tm of 131°C,[72] and it was the latter feature that raised concerns for potential off-target annealing, particularly after the authors showed that an AO with 2 mismatches was still able to induce targeted exon skipping.[72] We have shown that 2OMe AOs containing several mismatches could also induce targeted exon skipping *in vitro*, but this was only after application of high concentrations of AO, and skipping was not efficient compared to optimally designed AOs.[85]

Although earlier studies reported that PNAs may be of limited use as agents to induce exon skipping,[72, 100] this may again reflect limitations of delivery of the PNA into the nucleus, necessary for splice intervention. Recently, a report by Yin *et al.*[103] indicated that PNAs of 20 bases long could induce substantial exon 23 skipping in both *mdx* cells *in vitro* and *in vivo* after intramuscular injection. These authors were able to compare efficiencies of different oligomer chemistries and concluded that the PMOs were marginally more efficient than the PNAs. It should be noted that direct sequence comparisons were not reported.

One limitation of the PMO chemistry is poor uptake *in vitro*, unless either very high concentrations were used or the cells were encouraged to take up the PMO by scrape loading.[104] Sense strand oligonucleotide leashes, designed to anneal to a PMO directed at mouse exon 23 donor, allowed the uncharged PMO to be complexed with a cationic liposome, and induced targeted exon removal at concentrations three orders of magnitude lower than the uncomplexed PMO in cell culture.[100] Once the PMOs were taken up by the cells, high levels of exon skipping were maintained for the life of the cultures, as these uncharged compounds are not metabolized. The PMO chemistry does not show any overt toxicity *in vitro*, even when added to cultures at concentrations of 50μM. More importantly, no serious drug-related adverse events have been observed in 15 safety studies of 4 different PMOs, designed as anti-viral and metabolism modifying agents, involving approximately 350 individuals.[105]

It is not realistic to consider using cationic liposome preparations for repeated systemic delivery, based upon cost and more importantly, potential toxicity. The pluronic block co-polymer F127 was shown to enhance uptake of 2OMe AOs,[106, 107] and Wells *et al.*[108] showed enhanced AO delivery in the mouse using electroporation.

However, it is possible that systemic delivery may not be as great a challenge for PMOs as first anticipated. *In vivo* administration of a PMO was undertaken by injecting a cationic lipoplex composed of a PMO annealed to a sense strand leash, (1-5 ug) directed to the donor splice site of mouse dystrophin exon 23. As anticipated from *in vitro* studies, substantial dystrophin exon skipping was detected at the RNA and protein levels.[100] Immunofluorescence indicated that the induced dystrophin was correctly localized and the sarcolemmal complex was re-established. What was unexpected was that similar levels of exon skipping were induced after administration of an equivalent amount of PMO that was not annealed to the leash. It quickly became apparent that *in vivo* PMO uptake was much more efficient than anticipated from *in vitro* studies. Systemic studies followed and dystrophin expression could be detected in all tissues examined, except the heart.[109, 110] Further advances in PMO delivery came with conjugation to cell penetrating peptides, and even more substantial dystrophin expression was induced, using even lower doses of the PMO.[111] However, to date, the heart remains resistant to AO-induced exon skipping, prompting additional studies using different peptide tags and dosage regimens.

2.9 Alternative exon skipping strategies

Several approaches are being investigated as potential avenues to induce permanent exon skipping. Gene editing using chimeric RNA/DNA oligonucleotides (RDOs),[112, 113] single stranded oligodeoxynucleotides (ODNs),[114] and plasmid DNA has been reported.[115] If a base change could be introduced at a donor or acceptor splice site, the modified cell could maintain permanent exon skipping. Although the most common consequence of a splice site mutation is exon skipping, such as found in the canine model of muscular dystrophy,[39] there are many instances where a donor or acceptor mutation has led to the activation of a cryptic splice site, thereby causing intron retention or partial exon loss in the mature mRNA. We previously reported a case of germline mosaicism in a family with a defect in the exon 26 donor splice site.[116] This mutation did not lead to loss of exon recognition and skipping, but rather activation of a cryptic splice site downstream, with intron retention and an in-frame stop codon now in the mature mRNA. It may be difficult to predict the consequences of each splice motif mutation until it has been induced and validated in human cells. What is clear is that levels of induced gene correction reported are generally very low, and vary extensively

from one laboratory to another and this avenue of therapy is many years from the clinic.[117, 118]

Viral vectors are being developed to introduce expression cassettes that allow synthesis of antisense RNA sequences.[119, 120] Auxiliary sequences such as U1 and U7 are proposed to enhance accessibility of the AO to the splice site. Goyenvalle *et al.*[121] presented elegant work showing long-term dystrophin expression in the *mdx* mouse after introducing a viral construct carrying sequences annealing to the branch point of intron 22 and donor site of exon 23, linked to a modified U7 sequence under control of the U7 promoter. Dystrophin was readily detectable by western blotting and immunostaining 3 months after treatment. Should the appropriate construct be introduced into a stem or progenitor cell,[122] proliferation capacity could allow for potentially enhanced therapeutic benefits.

Autologous cell therapy is also being investigated to restore dystrophin.[123, 124] Cells were harvested and then transfected with a lentiviral construct, containing an exon skipping cassette designed to constantly generate RNAs to dislodge the target exon. The treated autologous cells were evaluated for exon skipping and transplanted into the tibialis anterior of *mdx*/SCID mouse. A few dystrophin positive myofibres were detected 10 weeks after transplantion,[123] and it appears that the low levels of dystrophin expression were caused by a combination of low exon skipping efficiency and poor viability of transplanted cells.

2.10 Clinical Trials

The first clinical trial to address a DMD-causing mutation by induced exon skipping involved a single patient with a frame-shifting deletion of exon 20. An oligomer, directed to exon 19, should restore the reading frame in this individual.[94] The treatment consisted of an ODN administered intravenously at a dosage of 0.5 mg/kg of body weight per week, for 4 weeks. Dystrophin protein was reportedly detected at very low levels by immunostaining of sections from the patient's biceps, 1 week after the last infusion. No western blot data was shown and the treatment failed to reduce serum creatine kinase levels. Although exon skipping was demonstrated in lymphocytes after the third and fourth treatments, only low levels of transcript missing exons 19 and 20 were found in the muscle biopsy after 4 treatments. In this report, preclinical testing was limited to one species, the *mdx* mouse, in which doses of 200 mg/kg were

administered by infusion. There were no adverse effects reported, but surprisingly neither were any exon skipping studies, since this particular oligomer matched the mouse dystrophin sequence perfectly and had been shown to induce mouse exon skipping *in vitro*.[85] Even more surprising, this compound had previously been reported to induce exon 19 skipping in the *mdx* mouse, but only after intraperitoneal injection.[125] Although a similar infusion protocol was used to administer this compound to a normal human volunteer for safety testing, no exon skipping analysis was reported.

This raises one of the fundamental problems with pre-clinical testing oligomers designed for dystrophin exon skipping. If this compound had efficiently dislodged exon 19 from the dystrophin pre-mRNA of the normal human volunteer, the reading frame will be disrupted, leading to reduced dystrophin expression, and in essence induce muscular dystrophy. This aspect is discussed in more detail below.

In addition, highly sensitive assays could lead to misinterpretation of exon skipping efficiency. Lymphocytes have been used to study illegitimate dystrophin expression,[126] where it has been estimated that one copy of dystrophin gene transcript occurs in about one thousand cells.[127] As very sensitive assays were required to detect these illegitimate transcripts, the low level of exon 19 skipping from the muscle biopsy is unlikely to reflect an accurate ratio of rescued muscle gene transcript. Several issues relating to dosage and route of administration, which were not properly addressed, highlight some limitations of this clinical trial.

A Phase I clinical trial in Leiden has now been completed.[102] A 2OMe AO designed to induce exon 51 skipping was injected into tibialis anterior muscles of 4 DMD patients. Exon 51 was chosen as the target for this study as its removal would correct the reading frame in more DMD individuals than any other exon, according to the Leiden muscular dystrophy database (http://www.dmd.nl/). Preexisting or fibroblast derived myogenic cells from patients were used for *in vitro* pre-screening of oligomer PRO051.[102] Four weeks after four intramuscular injections of 200 μg of PRO051 oligomer, running along a 1.5cm measuring line, muscle from the 8 to 12 year old participants were assessed for exon skipping at the RNA level and dystrophin restoration. Substantial dystrophin restoration was demonstrated in all patients by both immunostaining and western blot analysis.[102] This is the first evidence to conclusively support the potential of using AOs to restore dystrophin in DMD patients. Another trial has recently commenced injecting patients in the United Kingdom, also with the aim of

inducing exon 51 excision. However, there are several differences from the above study, including oligomer sequence,[89] oligomer chemistry and dosage, and the muscle to be treated.[101]

2.11 Future challenges

There is considerable optimism that AO-induced exon skipping may substantially reduce the progression and symptoms of DMD. However, a number of major challenges lie ahead. First, the classification of exon skipping as a gene therapy is regarded differently by regulatory agencies in different countries. We propose that the use of AOs to induce exon skipping should not be regarded as a form of gene therapy, since no permanent genetic changes are induced in the recipient. These oligomers cannot integrate into the DNA, and their mode of action interferes with gene expression, not the gene. In some respects, oligomers inducing exon skipping should be viewed no differently to a compound such as PTC124, which can suppress premature termination codons,[10] or any other molecule or antibody that blocks or modifies the function of a gene product. Upon cessation of the AO administration, the compounds will be either degraded by endogenous nucleases or gradually cleared from the system. No permanent changes will have been introduced, and while this may expedite some regulatory aspects of the work, it also poses a potential limitation, as the AOs will need to be re-administered at periodic intervals to maintain therapeutic levels of the induced protein.

The initial exon 51 skipping trials in Leiden and the United Kingdom will only provide proof-of-principle, with relatively limited information on safety being generated, since low doses of AOs of two chemistries are administered by an intramuscular injection. This mode of delivery cannot be considered to treat the entire body, although it may be possible to treat individual muscles in the hand, wrist, and forearm of older boys. This may preserve what little muscle is left and enhance their function.

The extent of the genomic deletion causing DMD will significantly influence the functionality of the AO-induced dystrophin isoform. The dystrophin isoform rescued by skipping of exon 51 in a DMD individual carrying a genomic deletion of exon 50 is likely to be more functional than the isoform induced in a patient whose gene lesion extended from exon 13 to 50. Targeted excision of exon 51 would restore the reading

frame in both deletions, but the missing coding region from the larger deletion would result in a greatly shortened dystrophin isoform. Genomic deletions in excess of 36 exons have generally been associated with a severe phenotype, regardless of the reading frame.[128] It is to be expected that different BMD-like dystrophin isoforms will have variable function, which would in turn influence the stability and rate of turn-over of the protein in muscle and presumably, the muscle fibres.

While different DMD patients with the same type of genomic mutation could be treated with the same AO preparation, the different dystrophic individuals may require oligomer dosage regimens that will be determined by their genetic background and nature of the dystrophin mutation. The influence of genetic backgrounds on manifestation of the same dystrophin mutation within one family has been reported.[50] Prescreening the target exons in recipients will then be essential to ensure there are no neutral DNA polymorphisms that could compromise AO annealing and hence excision of the targeted exon.

The cost of bringing a single drug to the market can be hundreds of millions of dollars.[129] In the case of drugs to treat common conditions, it would be expected that these may be used by millions of people. In these situations, extensive testing is mandatory to identify any adverse effects, particularly considering the number of individuals being exposed. The potential cost of bringing 12 different AOs, which would treat the majority of DMD deletion patients, to the market is staggering and will be beyond the capacity of any organizations other than the largest pharmaceutical companies. It should be noted that the `majority of deletion patients' would still only constitute about 60% of all DMD individuals, and a proportion of these would carry such large deletions, or the loss of crucial coding domains, such that exon skipping may not be a viable option.

The non-deletion DMD individuals will require many different AOs to address their mutations, as such lesions are scattered across the dystrophin gene. The concept of developing over 100 antisense compounds seems ludicrous, yet this must be considered if exon skipping is to be applicable to all amenable dystrophin mutations, especially since many of these defects occur in the large central rod domain. The loss of a single exon in the rod domain is expected to result in a dystrophin isoform of near normal function, since this is a variably dispensable part of the dystrophin protein. If AOs are to be used as a personalized genetic medicine, some compounds may be designed to

treat a mutation found in only a single family. We have shown that targeting dystrophin pseudo-exons with AOs can block their inclusion in the mature mRNA.[54, 130] Unlike restoring the reading frame around a genomic deletion or excising exons carrying nonsense mutations, AO-induced pseudo-exon suppression could lead to the production of a perfectly normal dystrophin. If the priority of targeted exon skipping is only based upon the frequency of mutations, then pseudo-exons would never be considered, despite being potentially the most responsive type of dystrophin gene defect.

Several PMOs of different sequences have been tested in animals, including mouse, rat, dog, and non-human primate for general toxicity and side-effects that may be associated with the backbone chemistry.[105] While safety studies of oligomers designed for viral gene suppression have been undertaken in normal human volunteers, similar safety trials cannot be considered for testing AVI-4658, a PMO designed to excise dystrophin exon 51. Evaluating AVI-4658 in normal human volunteers must be regarded as unethical due to unacceptable risks to the participants. If this compound works exactly as predicted, exon 51 would be removed from the normal dystrophin gene transcript, disrupting the reading frame and potentially inducing DMD. As shown in **Figure 2**, the consequences of exon 51 skipping will vary extensively between normal individuals and different DMD patients. In what should be a most amenable mutation (DMD Δ50), AVI-4658 would restore the reading frame around this single exon deletion, and allow synthesis of a dystrophin of near-normal length and function. The nature of induced dystrophin isoform will depend upon the extent of the primary gene deletion, hence potential benefits of exon skipping would be compromised by larger gene deletions; such as DMD Δ30-50 and Δ13-50. Thus, there is a broad spectrum of potential consequences of administering AVI-4658 to different individuals, ranging from no restoration of the reading frame in DMD patients with non-responsive mutations, induction of dystrophin isoforms of variable function depending upon the primary gene lesion, to inducing DMD in a normal individual by disrupting the dystrophin reading frame.

Although non-human primate studies may be more relevant, there are limitations, as there are no known primate models with dystrophin genomic deletions that would be restored by exon 51 skipping. Similarly, removal of a dystrophin exon could disrupt the reading frame and induce an adverse reaction associated with dystrophin deficiency, since the exon skipping compound would be working exactly as

it was designed to do. Animal testing of compounds designed for specific human dystrophin mutations can only supply limited information, and should be only undertaken if relevant data is generated.

There is currently no therapy available for DMD that addresses the missing or defective dystrophin. Although corticosteroids such as prednisolone or deflazacort have been shown a clear benefit in slowing muscle wasting,[131-133] mood swings, weight gain, stunted growth, brittle bone and cataracts have inevitably become acceptable side effects of the treatment. It is imperative that as many therapeutic compounds are made available to the DMD community in shortest possible time frame. DMD is a relentless progressive muscle wasting disorder that does not wait for regulatory approval, challenges in oligomer design, delivery, and production.

For the widespread implementation of oligomer induced splice intervention as a therapy for DMD, it may become necessary to regard induced exon skipping as a generic platform. If the first clinical trials show safety and efficacy in restoration of dystrophin expression after excising exon 51 from some patients, there must be a move to systemic administration and developing therapeutic dosage regimens. This is likely to take some considerable time, and may be confounded by the nature of the primary gene lesion and the genetic background of the patient. At the same time, additional exon targets must be considered to address other DMD mutations, and in this manner, sufficient safety data would become available that could allow different oligomer sequences of a particular class, for example PMOs, to be regarded as class-specific compounds.

Steroids, the current "gold standard" treatment to delay DMD progression, exert their effect through an unknown mechanism and have been available for decades. Despite this, there is still no consensus on the best dose and treatment regimen for steroids. Faced with the challenge of developing a personalized genetic intervention to address many different dystrophin mutations, it is most likely that establishing oligomer dosage regimens for individual DMD patients will prove to be an even greater challenge. Nevertheless, upon the demonstration of one mutation being amenable to exon skipping, we must make all efforts to expedite the application to as many different dystrophin mutations as possible.

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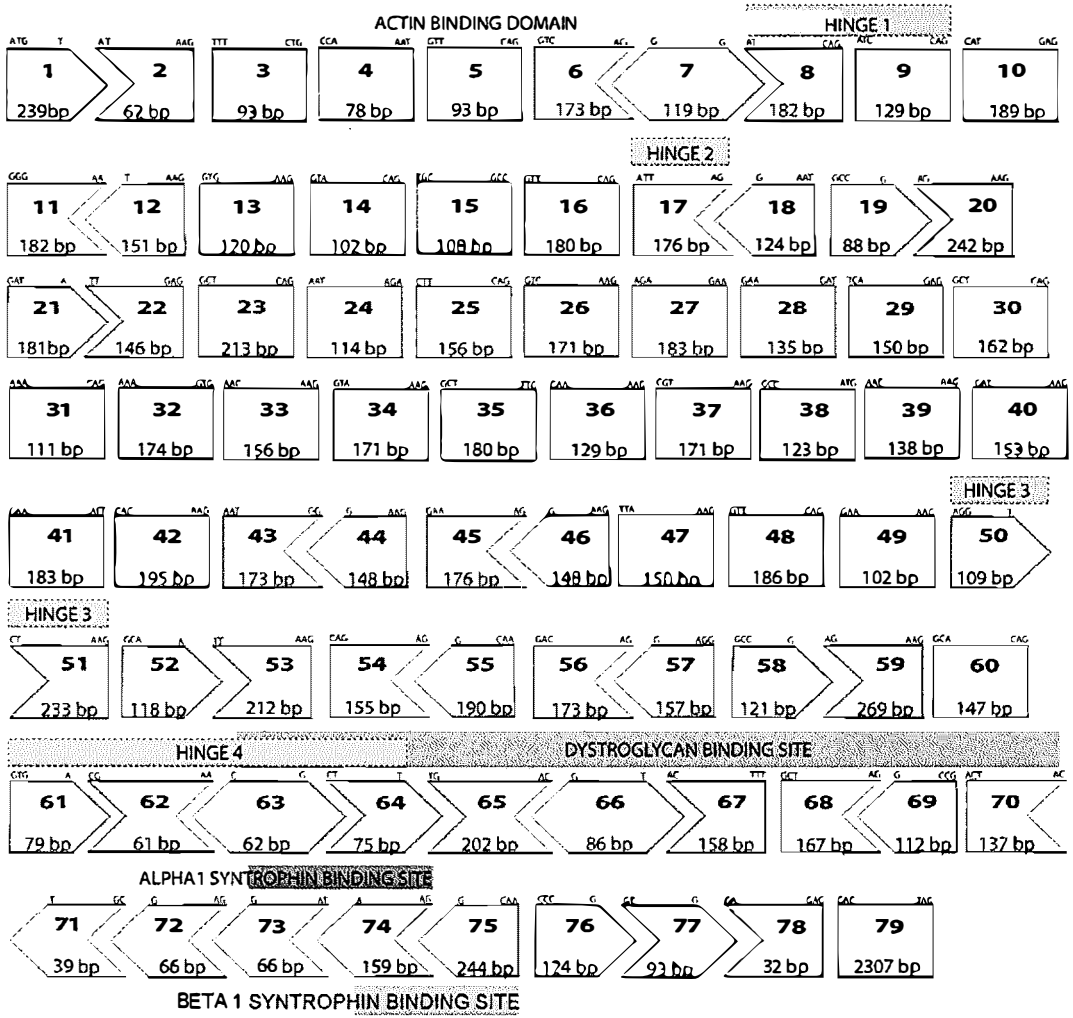

Figure 1: Structure of the dystrophin gene transcript indicating the reading frame and major functional domains.

Boxes represent in-frame exons whereas interlocking forward and reverse arrows and notches indicate codons spanning the exon:exon junctions. Junction codon sequences are shown above the exons.

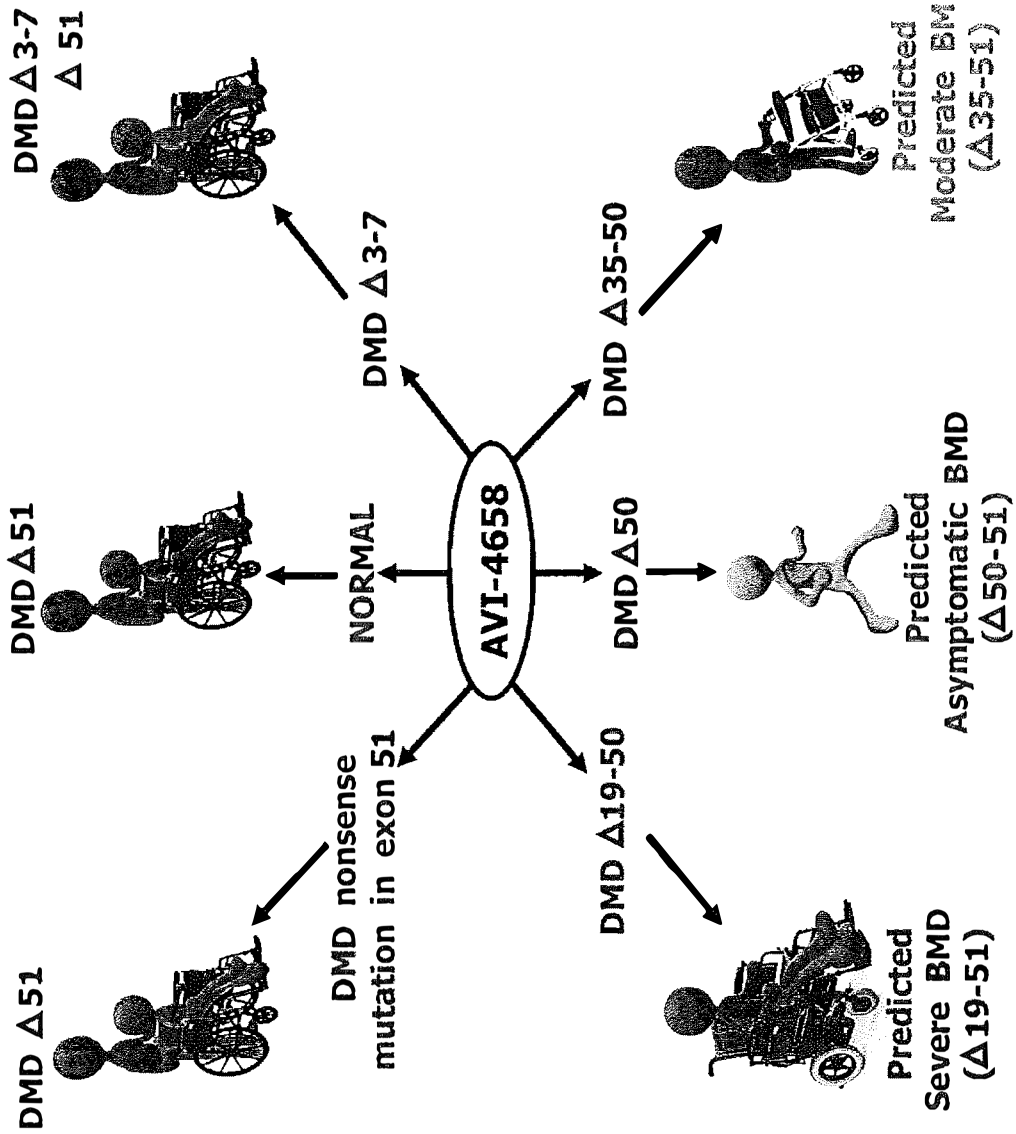

Figure 2: Potential consequences of AO induced exon 51 skipping in individuals with different dystrophin gene deletions.

Δn indicates deleted exons before and after treatment. Δ19-51, Δ 35-51 and Δ 50-51 are all in-frame transcripts and should lead to various BMD phenotype.

Table 1: Some examples of BMD deletions with associated comments on phenotype

Exonic Deletion	*Special comments*	*References*
3-9	*Playing competitive badminton at age 62 years.*	16
9-22	*High CK, myalgia but well developed musculature and no evidence of muscle weakness*	134
13-18	*Myalgia and cramps after normal activity*	135
13-41	*Very mild BMD*	136
17-47	*Source of the dystrophin mini-gene used in gene replacement studies*	34
17-51	*Mild BMD with congenital cataracts*	137
35-44	*Cramping after soccer or mountain climbing*	138
41-44	*Elevated CK, otherwise asymptomatic*	139
45-53	*Diagnosed age 60*	140
48	*Accidentally diagnosed in female, four affected male members then diagnosed with high CK only.*	141
50-53	*Elevated CK, otherwise asymptomatic*	139
It should be noted that very few BMD patients have been identified with in-frame deletion in the central rod domain involving exons 33-45.		142

CHAPTER 3

By-passing the nonsense mutation in the 4^{CV} mouse model of muscular dystrophy by induced exon skipping

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3.1 Abstract

Background Duchenne Muscular dystrophy (DMD), a severe neuromuscular disorder, is caused by protein-truncating mutations in the dystrophin gene. Absence of functional dystrophin renders muscle fibres more vulnerable to damage and necrosis. We report antisense oligomer (AO) induced exon skipping in the B6Ros.Cg-Dmd$^{mdx\text{-}4Cv}$/J (4^{CV}) mouse, a muscular dystrophy model arising from a nonsense mutation in dystrophin exon 53. Both exons 52 and 53 must be excised to remove the mutation and maintain the reading frame.

Materials and Methods A series of 2'-O-methyl modified oligomers on a phosphorothioate backbone (2OMeAOs) were designed and evaluated for the removal of each exon, and the most effective compounds were then combined to induce dual exon skipping in both myoblast cultures and *in vivo*. Exon skipping efficiency of 2OMeAOs and phosphorodiamidate morpholino oligomers (PMOs) was evaluated *in vitro* and *in vivo* at the RNA and protein level.

Results Compared to the original *mdx* mouse studies, induction of exon skipping from the 4^{CV} dystrophin mRNA was far more challenging. PMO cocktails could restore synthesis of near-full length dystrophin protein in cultured 4^{CV} myogenic cells and *in vivo*, after a single intramuscular injection.

Discussion By-passing the protein truncating mutation in the 4^{CV} mouse model of muscular dystrophy could not be achieved with single oligomers targeting both exons and was only achieved after the application of AO cocktails to remove exons 52 and 53. As with previous studies, the stability and efficiency of PMOs proved superior to 2OMeAOs for consistent and sustained protein induction *in vivo*.

Keywords Exon skipping; B6Ros.Cg-Dmd$^{mdx\text{-}4Cv}$/J (4^{CV}) mouse; Antisense oligomer, Duchenne muscular dystrophy; morpholino.

3.2 Introduction

Duchenne muscular dystrophy (DMD), a devastating neuromuscular disorder characterised by progressive muscle wasting, and proximal muscle weakness, arises from the absence of functional dystrophin. Dystrophin links the actin cytoskeleton to the extracellular matrix via a complex of proteins embedded in the sarcolemma and plays a pivotal role during muscle contraction (for review see [1-3]). Loss of dystrophin renders muscle fibres vulnerable to membrane damage during contraction. Progressive loss of muscle fibres, with inflammatory cell infiltration and fibrosis eventually overwhelms the regenerative capacity of the muscle.

The human dystrophin gene is the largest known, and the major muscle isoform consists of 79 exons, spanning 2.4 million bp. Most mutations in DMD patients are intragenic deletions or duplications, accounting for about 60% and 8% of all DMD patients, respectively.[4-7] Point mutations, including nonsense and splice motif mutations, as well as small insertions/deletions that disrupt the reading frame, are responsible for 25 to 35% of all cases.[7, 8] DMD mutations typically disrupt the reading frame, thereby preventing synthesis of functional dystrophin. Becker muscular dystrophy (BMD) is also caused by mutations in the dystrophin gene, but this milder allelic condition is generally caused by gene defects that do not disrupt the reading frame and allow production of shorter, but partially functional protein. Depending upon the position and nature of the mutation, some cases of BMD may only be diagnosed late in life, and present with very mild or no symptoms,[9, 10] while others may present as borderline DMD and lose ambulation around the age of 15.[11, 12]

AO-induced exon skipping studies initially targeted different splice motifs of exon 23 in the muscular dystrophy mouse model (*mdx*), with the aim of restoring protein expression.[13, 14] The defect in the *mdx* mouse is a naturally occurring nonsense mutation in dystrophin exon 23. Despite limitations, including a mild clinical phenotype, the *mdx* mouse has been widely used in developing potential therapies for DMD, including exon skipping,[15-22] gene and cell replacement (for review see [23, 24]), and premature translation termination suppression.[25]

In this study, the B6Ros.Cg-Dmd$^{mdx\text{-}4Cv}$/J (4^{CV}) muscular dystrophy mouse,[26] carrying a nonsense mutation in exon 53 of the dystrophin gene, was used to evaluate AO-induced dual exon skipping in a region of the dystrophin gene within the major

human dystrophin deletion hot-spot. By-passing the 4^{CV} mutation, and maintaining the reading frame, requires removal of both exons 52 and 53 from the mature dystrophin gene transcript. A series of AOs were designed and evaluated for the removal of each exon, and the most effective compounds were then combined to induce dual exon skipping in both myoblast cultures and *in vivo*. AOs of two different chemistries, 2'-O-methyl modified oligomers on a phosphorothioate backbone (2OMe), and phosphorodiamidate morpholino oligomers (PMOs) conjugated to a cell-penetrating peptide (P007)[27, 28] were compared.

3.3 Materials and methods

3.3.1 AOs and primers

AO nomenclature is based on that described by Mann et al., 2002.[18] Sequences and composition of AO treatments are described in Table 1. 2OMe AOs were synthesized on an Expedite 8909 Nucleic Acid synthesizer using the 1 μmole thioate synthesis protocol. AOs were designed to anneal to either exonic sequences or exon/intron junctions of mouse dystrophin exons 52 or 53. PMOs conjugated to an arginine-rich, cell penetrating peptide (P007)[27, 28] were synthesized by AVI BioPharma (Corvallis, OR, USA). Primers for RT-PCR and sequencing analysis were synthesized by Geneworks (Adelaide, Australia) and are listed in supplemental information.

3.3.2 Animals

4^{CV} (B6Ros.Cg-Dmd$^{mdx\text{-}4Cv}$/J) congenic mice, obtained from the Jackson Laboratory (Maine, USA), were raised and supplied by the Animal Resources Centre, Murdoch, Western Australia. Animal housing and transport followed guidelines from National Health and Medical Research Council (Australia). The use of animals was approved by the Animal Ethics Committee of University of Western Australia (approval number 03/100/572).

3.3.3 Cell culture and AO transfection

Immortalized *mdx H2K^{b}-tsA58* mouse cells (*H-2K mdx*) were propagated and transfected as described previously.[29] Primary myoblast cultures were prepared from 2 to 4 days old 4^{CV} pups and the procedure was adapted from Rando *et al.*[30] Limb muscles from 4 pups were dissected, homogenized, and incubated at 37°C

for 30 minutes with dissociating enzyme mix containing 2.4 units/ml dispase (Invitrogen, VIC, Australia), 5 mg/ml collagenase Type II (Invitrogen), and 2.4 mM $CaCl_2$ in DMEM (Invitrogen). After centrifugation, the cell pellet was added to a 75 cm^2 tissue culture flask with 10 ml of proliferative media and incubated for one hour. Non-adherent cells were removed and seeded into 75 cm^2 tissue culture flasks coated with 100 µg/ml matrigel. When nearly confluent, cells were seeded into 24 well-plates coated with 50 µg/ml poly-D-lysine solution and matrigel, and incubated for 48 hours before transfection. Duplicate wells were transfected with 2OMeAO lipoplexes using Lipofectin (Invitrogen) at a ratio of 2:1 Lipofectin to AO. Briefly, Lipofectin was mixed with OptiMEM (Invitrogen) to a final volume of 200 µl and incubated for 30 minutes at room temperature. The 2OMeAO, which had been diluted to 200 µl in OptiMEM, was then combined with Lipofectin:OptiMEM and the mixture incubated for a further 30 minutes, before addition of OptiMEM to a final volume of 1 ml and subsequent addition of 500 µl aliquots to each well. Transfected cells were incubated for 48 hours before RNA was extracted for analysis.

3.3.4 Intramuscular administration

Oligomers, in physiological saline, were injected into *tibialis anterior* (TA) muscles at doses indicated. Each experiment included at least one saline only injection as a negative control. The animals were anesthetized and sacrificed by cervical dislocation at indicated time points after the injection, and muscles were removed and snap-frozen in pre-cooled isopentane, before being sectioned and prepared for RNA and protein studies.

3.3.5 RNA extraction, RT-PCR analysis and DNA sequencing

RNA was harvested from *H-2K mdx* cultures, 4^{CV} cultures and frozen sections tissue block using Trizol (Invitrogen), according to the manufacturer's protocol. One-step RT-PCR was undertaken using 120 ng of total RNA as template, in a 12.5 µl reaction for 30 cycles of amplification. After the reverse transcription step for 30 minutes at 55°C, the reaction was heated to 94°C for 2 minutes before the primary thermal cycling rounds of 94°C for 40 seconds, 60°C for 1 minute, and 68°C for 1 minute. Nested PCR was then carried out on 1 µl of the primary amplification reaction using AmpliTaQ Gold (Applied Biosystems). Cycling conditions for the secondary

PCR were 94°C for 6 minutes to activate the polymerase, followed by 20 cycles of 94°C for 40 seconds, 60°C for 1 minute, and 72°C for 1 minute. PCR products were separated on 2% agarose gels in TAE buffer and the images captured on a CHEMISMART-3000 (Vilber Lourmat, Marne-la-vallee, France) gel documentation system. Bands of interest were re-amplified directly from the agarose gel,[31] and the sequencing templates were purified using UltraClean spin columns (Mobio Laboratories, CA, USA) and then sequenced on a 3730 ABI DNA sequencer using BigDye v3.1 terminator chemistry (Applied Biosystems).

3.3.6 Western blot analysis

Protein extracts were prepared as weight per volume of treatment buffer containing 125 mM Tris/HCl, pH 6.8, 15% sodium dodecyl sulphate, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM dithiothreitol, bromphenol blue, and protease inhibitor cocktail (Sigma) to cell pellet or mouse muscle cryostat sections. Samples were then vortexed and sonicated briefly, heated at 95°C for 5 minutes, before electophoretic fractionation on a 4 to 10% SDS gradient gel at pH 8.8 with 4% stacking gel, pH6.8. Densitometry of myosin bands after coomassie blue staining was undertaken to facilitate equivalent protein loading. Extracts from AO treated cultures or muscle cryosections from treated animals (2.75 mg) and control muscle (0.275 mg) were loaded onto a second PAGE gel for Western blotting. Proteins were transferred from the gel to nitrocellulose membranes (Amersham Biosciences, Castle Hill, Australia) overnight at 18°C, at 290 mA. Dystrophin was visualised using NCL-DYS2 monoclonal anti-dystrophin antibody (Novocastra, Newcastle-upon-Tyne, UK) as described previously.[21] Images were captured on a Vilber Lourmat CHEMISMART-3000 gel documentation system. Percentage of dystrophin restoration was calculated according to dystrophin expression in control cells after normalization for myosin loading.

3.3.7 Tissue preparation and immunofluorescence

TA muscles were taken from mice and snap-frozen in isopentane, pre-cooled in liquid nitrogen. Dystrophin was detected in 6 μm unfixed cryostat sections using NCL-DYS2, an antibody that reacts strongly with C-terminus of dystrophin. Immunofluorescence was performed using the Zenon Alexa Fluor 488 labeling kit (Invitrogen), according to protocol recommended by manufacturer, except for the initial

fixation step. The primary antibody was diluted at 1:10 and sections were counterstained with Hoechst (Sigma) at the dilution of 1:10,000 to visualize nuclei. Sections were viewed under Olympus IX 70 inverted microscope and images were captured on an Olympus DP70 digital camera.

3.4 Results

3.4.1 Single exon targeting

A panel of AOs was designed to anneal to obvious splicing motifs, including the acceptor and donor splice sites, and potential exonic splicing enhancers (ESEs) of both exons 52 and 53. Although designed to predict human ESEs, the web-based ESE prediction program, ESEfinder 3.0,[32, 33] was used to identify putative ESEs in mouse dystrophin exons 52 and 53. **Figure 1** shows the predicted ESEs of exons 52 and 53 and the relative annealing positions of the AOs (sequences provided in Table 1) designed to induce exon removal. Six AOs, designed to excise dystrophin exon 52, were evaluated initially in *H-2K^{b}-tsA58 mdx* (*H-2K mdx*) immortalised myogenic cells.[34] The use of these immortalized cells reduced animal usage and, since the 4^{CV} mutation in exon 53 did not alter dystrophin splicing patterns, it was assumed that design in one mouse strain should be valid for another. Subsequent experiments validated this approach. According to ESEfinder, the nonsense mutation in dystrophin exon 53 occurs in two predicted ESE motifs that are only just above the threshold: an SF2/ASF motif (score 2.187/threshold 1.956) and an SRp40 motif (score 3.042/threshold 2.67).

None of the AOs targeting the acceptor or donor sites of exon 52 consistently induced removal of the target (**Fig 2a**), whereas the AOs annealing between the coordinates +17+51 efficiently excluded exon 52 from the mature dystrophin mRNA (**Fig 2b**). Subsequent titration studies comparing AOs 1 and 4 indicated the latter compound was more efficient at inducing exon 52 removal at lower transfection concentrations, although there was little exon 52 skipping induced at 25 nM (data not shown). Consequently, AO4 was selected for further studies.

Excision of exon 53 proved to be a greater challenge, and although numerous AOs were designed and evaluated, no single AO tested efficiently excluded exon 53 from the mouse dystrophin mRNA. While many AOs failed to dislodge exon 53, as shown in **Figure 2c**, others removed exon 53 in addition to both exons 53+54 (**Fig 2d**).

Two AOs activated a cryptic splice site and resulted in partial exon 53 loss (Figure 2e). Thirteen different combinations of non-overlapping AOs were then assessed and one AO cocktail was developed to consistently induce specific exon 53 skipping (**Fig 2f**).

3.4.2 Exon 52+53 skipping: 2OMe AOs

As the relative efficiencies of excision of the target exons differed, it was necessary to evaluate combinations of the optimized AOs directed to exons 52 and 53, shown in **Table 2.1**. Four 2OMeAOs (4, 8, 9, 13) were combined at ratios indicated (A1, A2, A3, A4, and A5) and used to transfect 4^{CV} cultured primary myoblasts (**Fig 3a-c**). The identity of the transcript missing exons 52 and 53 was confirmed by DNA sequencing (**Fig 3d**). At 2 days after transfection, the A2 and A3 cocktails appeared marginally more effective at excising both exons 52 and 53, as shown by the absence of the full-length product (**Fig 3a-c**), hence the A2 cocktail was selected for further studies.

Although RT-PCR studies indicated substantial exons 52+53 skipping at days 3 and 5, only a trace of shortened transcript could be detected at days 8 and 9 after treatment (data not shown). No detectable dystrophin protein was observed in treated cultures at any time point. RNA and protein were analysed at day 14 after two transfections at days 0 and 9, and no exon skipping or restored dystrophin protein were observed (data not shown).

A total of 100 μg of the 2OMeAO A2 cocktail, was complexed with F127 and administered to 4^{CV} mice through intramuscular or intraperitoneal routes. Animals were sacrificed 3, 5, and 7 days after injection and RNA from the diaphragm and TA muscle was assessed for exon skipping. Although sporadic exon 52 and 53 skipping was detected in the TA muscle, no substantial dystrophin protein restoration could be demonstrated by either immunofluorescence or western blot analysis (data not shown).

3.4.3 Induced exon 52 and 53 skipping with phosphorodiamidate morpholino oligomers

We had previously found that while 2OMeAOs were well suited for AO design and short-term *in vitro* RNA studies, restoration of dystrophin protein in 2OMeAO treated cultures was more problematic.[35] AOs 4, 8, 9 and 13 were prepared as PMOs, coupled to the cell penetrating peptide P007. These oligomers did not induce any exon skipping when applied individually (data not shown). The combination was evaluated

in 4^{CV} myoblast cultures, and as with the 2OMe AOs, different ratios of PMOs were assessed. The PMO cocktails B2, B3 and B4 induced the transcript missing both target exons at concentrations as low as 0.5 μM *in vitro*. The B2 and B3 PMO combinations comparable to the A2 and A3 2OMe combinations appeared to be the most effective mixtures, and were selected for further study (**Fig 3e-g**). Both the B2 and B3 cocktails were able to induce pronounced exon 52 and 53 skipping, 2 weeks after *in vitro* transfection at a concentration of 40 μM (**Fig 3h**), consistent with the appearance of induced dystrophin, as determined by western blotting (**Fig 3i**). Normalization of dystrophin according to myosin densitrometry indicates the B2 and B3 cocktails induced 11 and 8% of normal levels of dystrophin respectively (**Fig 3i** and Supplementary data 2.1).

3.4.4 Evaluation of off-target effects

To confirm specificity of the multi-oligo cocktail on dystrophin expression, RT-PCR was undertaken across the dystrophin gene transcript using 5 sets of nested primer pairs (supplementary data) covering exons 13 to 70 region (**Fig 4a-e**). **Figures 4a to e** represented amplified segments of the dystrophin transcript. Although sporadic "revertant" transcripts were detected in PMO cocktail treated cultures, the numbers of alternatively spliced transcripts were not greatly different from those in 6 untreated cultures (**Fig 5**).

3.4.5 *In vivo* studies

A single injection of 40 μg of each of the PMO cocktails, at ratios shown in Table 2.3, was made into the TAs of 4^{CV} mice. Two weeks after injection, RT-PCR on RNA extracted from injected 4^{CV} muscle demonstrated exon 52 and 53 skipping in all cocktail-treated samples (**Fig 6a**) with western blotting indicating dystrophin expression to be approximately 5 to 7%, based upon normalization of loading (supplementary information). Consistent with the RNA studies, dystrophin immunofluorescence on sections from the *TAs* of 4^{CV} mice treated with PMO cocktails C1 and C2, also showed dystrophin-positive fibres (**Fig 7a, b**).

3.5 Discussion

Oligomer design, evaluation of different chemistries and systemic delivery protocols have been extensively studied in the *mdx* mouse model of muscular dystrophy.[14, 18-21, 36, 37] We describe the application of AO-induced dual exon skipping

to address the primary gene lesion in another mouse model, the 4^{CV} mouse, whose nonsense mutation should be by-passed with the excision of exons 52 and 53. This model may be regarded as of greater relevance to the human condition, since the targeted exon skipping is induced within the major hotspot for dystrophin gene deletions.[38]

Acceptor and donor splice sites were initially considered obvious targets for AO-induced exon skipping, and consistent exon 23 removal was achieved with the first oligomer targeting the donor splice site.[14] However, a comprehensive study targeting all human dystrophin exons indicated donor splice sites are rarely the optimal or preferred targets, and the majority of preferred targets appear to be intra-exonic motifs.[39] Indeed, it appears that if a pre-mRNA site is not amenable to oligomer intervention, the application of a panel of oligomers to microwalk across that motif, or altering AO length or chemistry, is unlikely to achieve acceptable (or detectable) levels of exon exclusion.[40] Similar trends were observed for the 4^{CV} mouse dystrophin exons 52 and 53, where individual oligomers annealing to either the donor or acceptor splices sites failed to induce any targeted exon skipping. Two overlapping oligomers, AOs 1 and 4, targeting intra-exonic motifs within exon 52, were identified that induced substantial skipping and titration studies indicated AO 4 was slightly more efficient at lower transfection concentrations.

Mouse dystrophin exon 53 proved to be much more challenging to dislodge from the mature mRNA. Eighteen oligomers were designed to target acceptor and donor splice sites, as well as intra-exonic motifs predicted by ESEfinder.[32, 33] Although this program is based upon human sequences, the strong homology between mouse and human dystrophin sequences was considered sufficient justification to use this program as a starting point in AO design. Initial AO design was undertaken in an immortalized *mdx* mouse myogenic cell line, while awaiting establishment of the 4^{CV} mice colony. The sequences of dystrophin exon 52 were identical in both strains, and similar identified splice patterns were generated with AO4 in both cell lines. Similarly, AOs that led to exon 53 skipping in the *mdx* cell line exhibited identical patterns in cultured cells from the 4^{CV} mouse. Since no aberrant splicing was observed involving exon 53 in untreated 4^{CV} cultures, it was presumed that the mutation did not influence splicing. This is consistent with the mutation occurring in two putative ESEs that were just above the threshold for SF2/ASF and SRp40. Once the 4^{CV} colony was established, all

subsequent experiments were undertaken in these cells.

The majority of compounds targeting exon 53, when used individually were either ineffective, activated a cryptic splice site (loss of 78 bases from the end of exon 53), or led to the production of dystrophin gene transcripts missing exons 53+54, in addition to exon 53 alone (**Fig 2d-f**). Interestingly, AO 7, which anneals to exactly the same co-ordinates identified as an optimal target to dislodge human dystrophin exon 53, induced mouse dystrophin transcripts missing exons 53+54, in addition to exon 53 excision. Several other AOs overlapping AO 7 also induced this pattern of exon removal, implying that this area may be involved in coordinated processing of both exons during pre-mRNA splicing, at least in mice. Targeting exon 53 for excision from the human dystrophin gene transcript only resulted in specific target removal, highlighting some limitations in extrapolation of oligomer design between homologous genes of different species.[39] The loss of an exon flanking that targeted for excision has been reported previously. The AO targeting the *mdx* mouse exon 23 for removal also induced dystrophin gene transcripts missing exons 22+23, an out-of-frame transcript that cannot lead to dystrophin production.[36] Similarly, transcripts missing exons 53+54 are out-of-frame, even when exon 52 was omitted and were thus considered undesirable.

As we had previously observed that some combinations of apparently inactive AOs were able to induce highly efficient exon skipping,[41] various AO cocktails targeting exon 53 were evaluated. A combination of AOs 8 and 9, targeting the acceptor and donor sites respectively, was able to induce some exon 53 skipping, although not at a consistent and satisfactory level. Subsequently, AOs 8 and 9 were combined with other AOs (7, 10, 11, 12, 13, 14, 16) to identify a cocktail capable of inducing specific exon 53 skipping. When AO 13 was combined with AOs 8 and 9, efficient and specific exon 53 removal could be induced after transfection at low total AO concentrations. The mixture of AOs 8 and 13 generated the same pattern induced by AO 13 alone, indicating no advantage, while the combination of AOs 9 and 13 only induced inconsistent exon 53 excision. This 3 AO combination was necessary for both efficiency and specificity of exon skipping.

Since exon 53 was removed from the mature mRNA at a higher efficiency than exon 52, attempts were made to balance the ratio of AO 4, targeting exon 52, with AOs 8, 9 and 13, to maximize induction of in-frame transcripts. When equimolar amounts of all four oligomers were applied, the full-length transcript was observed, in addition to

dystrophin mRNAs missing exon 53 and 52+53. Altering the composition of the cocktails with increased proportions of AO 4 resulted in decreased amounts of full-length product and a higher proportion of in-frame transcripts missing both exons 52+53. Transcripts missing only exon 52, another out-of-frame mRNA was also observed.

Although, substantial exons 52+53 deleted transcripts were detected at days 3 and 5 after transfection with 2OMe AOs, no detectable dystrophin was observed by western blot analysis (data not shown). Only trace amounts of exon skipping could be observed 8-14 days after transfection, and this also did not generate sufficient dystrophin detectable by western blotting (data not shown). It was assumed that this limitation arises from the uptake and turn-over of the 2OMeAOs in cells. The 2'-O-modified oligomers on phosphorothioate backbone increase the oligomers resistance to nuclease degradation, but unlike PMOs which are not metabolized, 2OMeAOs are gradually broken down by nucleases.[42] This was most apparent in a time-course of 2OMeAO-induced exon skipping in cultured cells, where there was substantial exon skipping 24 hours after transfection, but this declined substantially over the next 9 days.[43] In contrast, substantial PMO-induced exon skipping transcript had been detected with less intermediate product at 14 days after transfection, *in vitro*.[44] By-passing the dystrophin gene lesion and inducing dystrophin detectable by western blotting was very inconsistent in canine cultured cells from the GRMD model of muscular dystrophy and human dystrophic muscle explants.[35, 44] However, upon the application of PMOs of the same sequences, efficient exon skipping could be induced and maintained *in vitro* or *ex vivo* to allow sufficient dystrophin synthesis to be readily detected by western blotting at 7 days after transfection.[35]

The application of any oligomer to a cell has the potential to cross react with other sequences and/or exert non-specific effects. The use of AO cocktails would potentially increase this risk and although a detailed examination of other genes has not been undertaken, we showed that the cocktail only induced targeted changes in the dystrophin mRNA. We had noticed in other studies that the addition of oligomers seems to marginally increase the appearance of alternatively spliced transcripts.[45] However, the application of 4 oligomers did not greatly alter the incidence of the alternative transcript in treated cells compared to untreated cultures.

The application of PMOs, coupled to an arginine rich cell-penetrating peptide, induced more robust exon skipping *in vitro* and *in vivo* in 4^{CV} cells than the equivalent 2OMeAOs. As we had previously shown that a hierarchy of exon skipping efficiency induced by 2OMeAOs was also seen when the same sequences were evaluated as PMOs,[46] the most effective mixtures of 2OMe and PMO AO appeared comparable in the 4^{CV} model, although there was a minor shift in the relative proportions of exon excised transcripts. The optimal 2OMeAO cocktails induced removal of roughly equivalent amounts of exons 52 and 52+53, while the equivalent PMO combinations generated transcripts missing exon 53 as the predominant transcript and, exons 52+53. Approximately 10% of restored dystrophin protein was induced according to calculation of band densitrometry of western blot analysis.

PMO cocktails, consisting of a total of 40 μg of the oligomers directed to exons 52 and 53, were administered into the *TAs* of 4^{CV} mice by a single intramuscular injection. Two weeks later, dystrophin was observed by dystrophin immuno-fluorescence after C1, C2, or C3 treatment. Calculation of band densitometry from Western blot analysis demonstrated approximately 5-10% of restored dystrophin protein after C1, C2, or C3 treatment. Further studies in this area may provide additional information to understand and to refine more efficient combinations for AO-induced dual exon skipping.

In summary, there still appears to be no consistent pattern in AO design for targeted exon skipping. Masking an obvious splice motif, such as the donor splice site that proved so effective in the *mdx* mouse, was ineffective in excising different exons in another mouse model of muscular dystrophy. AO cocktails were essential for by-passing the mutation in the 4^{CV} mouse in terms of both specificity and efficient exon removal. Consistent with other studies, the PMOs appeared superior to 2OMeAOs, especially *in vivo*. Long-term systemic treatment of the 4^{CV} mouse is now underway to establish an appropriate delivery regimen that would be best suited for dual exon skipping, in a region of the dystrophin gene located within the human deletion hotspot.

3.6 References

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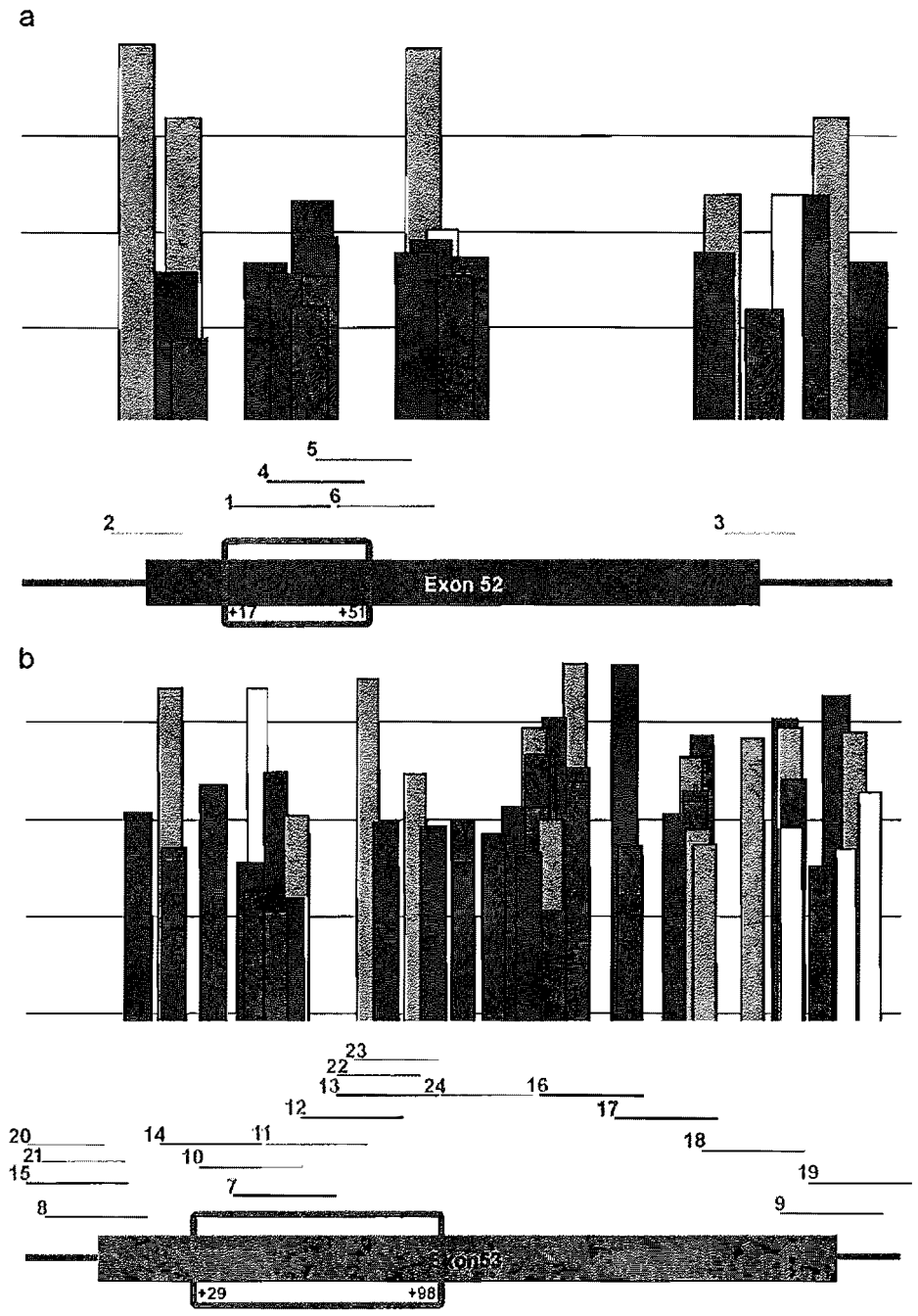

Figure 1: Diagramatic presentation of predicted ESEs; (a) mouse exons 52; and (b) 53.

Relative annealing coordinates of oligomers are indicated. Vertical bars indicate the five ESEs predicted to be binding sites of SR proteins (viz. SF2/ASF (red), SF2/ASF (IgM-BRCA1) (pink), SC35 (navy blue), SRp40 (light green) and SRp55 (yellow)). Threshold scores were set at default values as specified in ESE finder.[32, 33]

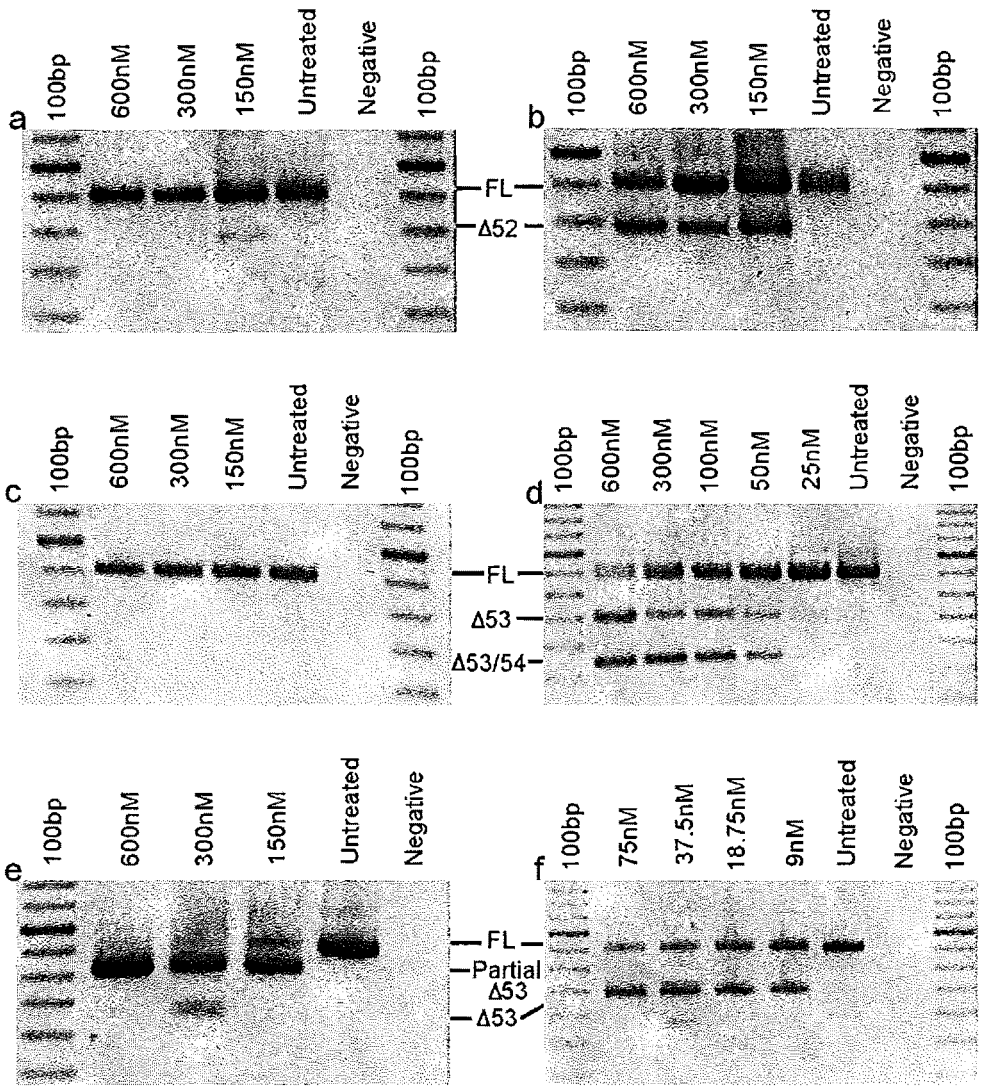

Figure 2: Induced single exon skipping in *H-2K mdx* cells.

(a) Transcripts resulting after transfection with AO 2 (typical of that obtained after transfection with AOs 3,5,6); (b) Transcripts resulting after transfection with AO 4 and AO 1; (c) Transcripts resulting after transfection with AO 8 (typical of that obtained after transfection with AOs 9,14,15,16,19-24); (d) Transcripts resulting after transfection with AO 7 (typical of that obtained after transfection with AOs 7,10-13); (e) Transcripts resulting after transfection with AO 17; (f) Transcripts resulting after transfection with cocktail AOs 8+9+13.

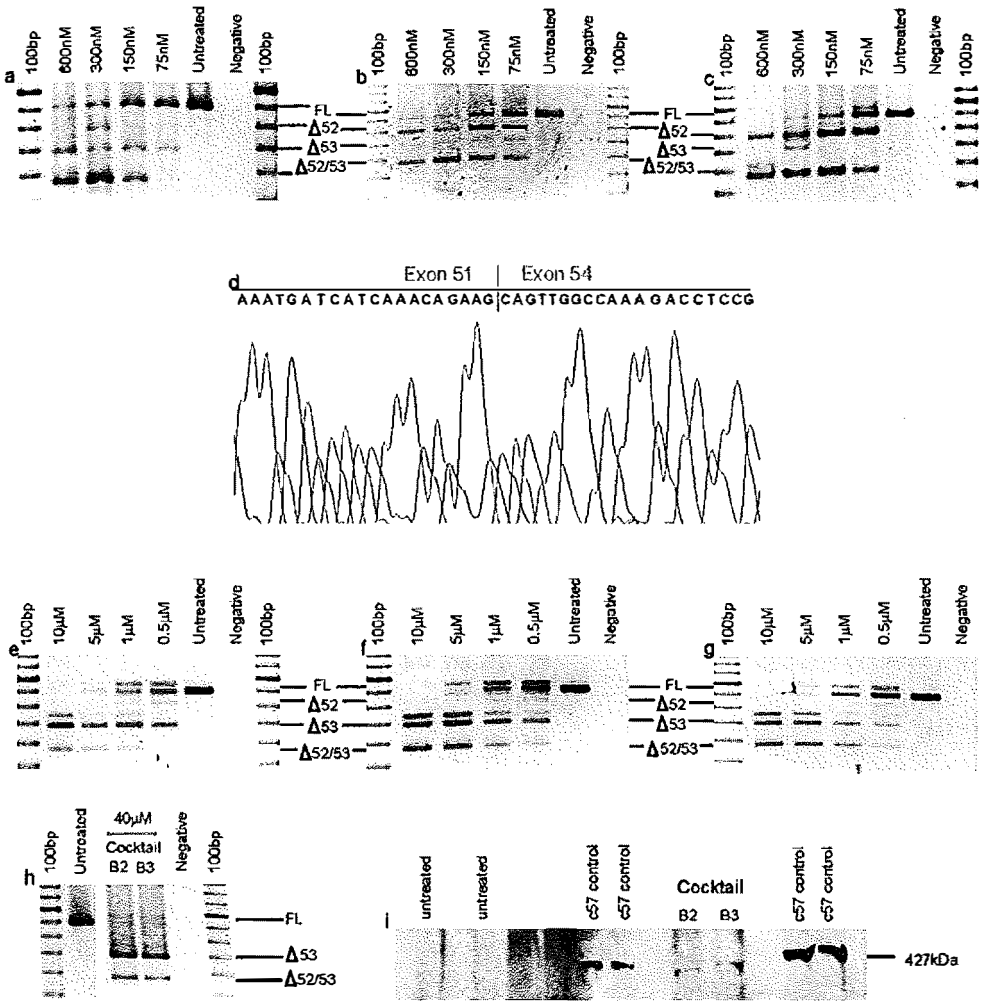

Figure 3: Induced exon skipping in 4^{CV}cells after transfection with 2OMeAO cocktails.

(a) A1; (b) A2; and (c) A3; (d) DNA sequencing chromatogram confirming precise splicing of exon 51 to 54. Transcripts resulting after transfection with (e) B1; (f, h) B2; (g, h) B3 in 4^{CV} cultures ; (i) Western blot analysis from 4^{CV}cultures treated with B2 and B3 PMO cocktails.

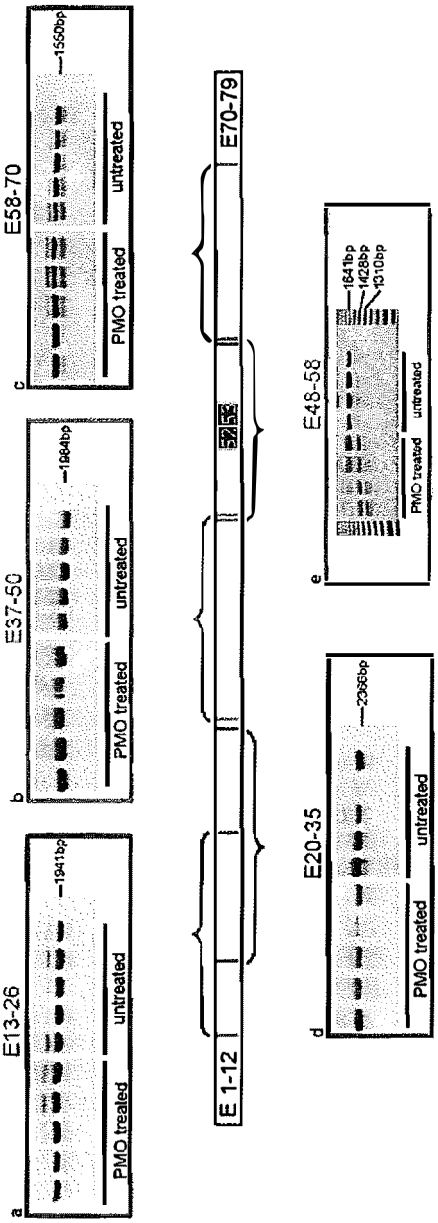

Figure 4: RT-PCR analysis of dystrophin transcripts in untreated and PMO treated 4^{cv} cultures across (a) exons 13-26; (b) exons 37-50; (c) exons 58-70; (d) exons 20-35; (e) exons 48-58.

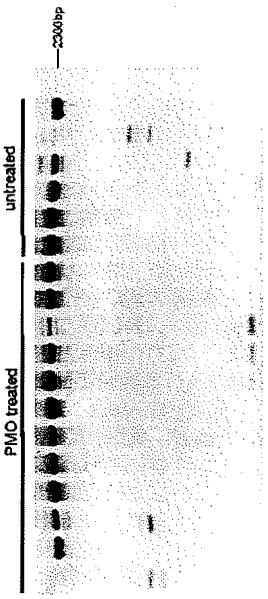

Figure 5: RT-PCR analysis of dystrophin transcripts in untreated and PMO treated 4^{cv} cultures across exons 20-35 indicating alternatively spliced dystrophin transcripts.

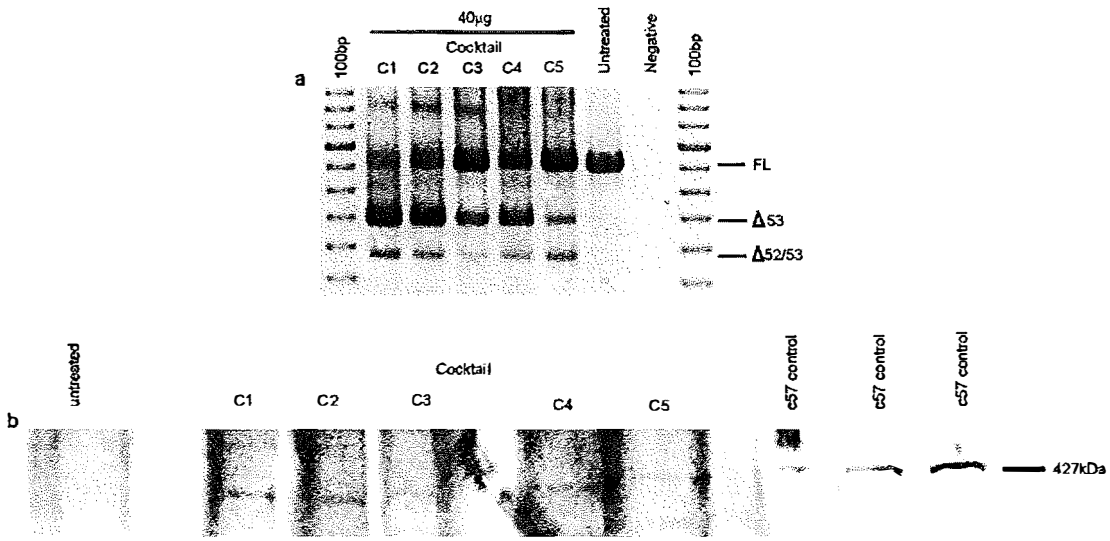

Figure 6: (a) RNA studies; and (b) western blot analysis of muscle extracts from the 4^{CV} mice injected (IM) with PMO cocktails (C1 to C5).

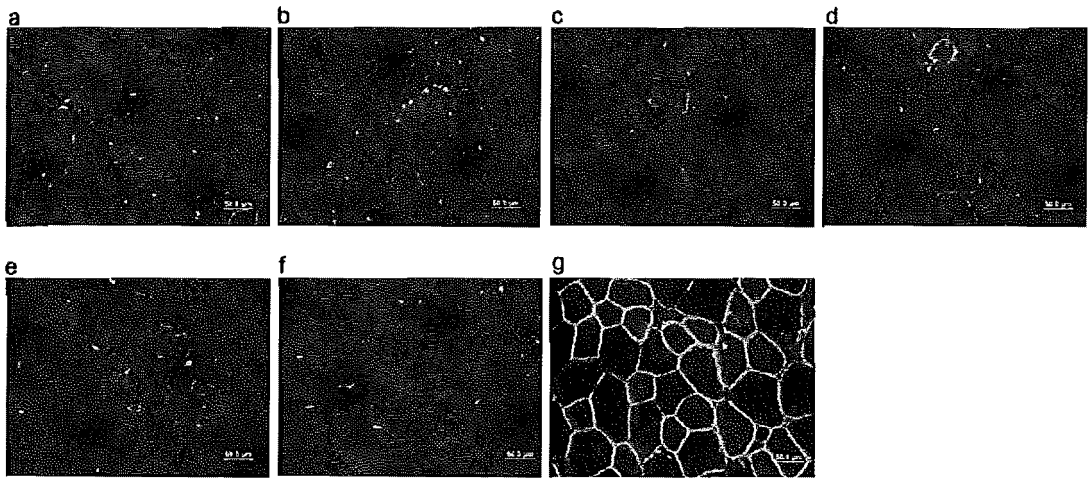

Figure 7: Dystrophin immunofluorescence on muscle cryosection from 4^{CV} mice treated with cocktails (a) C1; (b) C2; (c) C3; (d) C4; (e) C5; (f) saline; and (g) untreated c57BL/10ScSn

Table 1 : The lengths, annealing coordinates, sequences and GC content (number and %) of the 24 selected AOs targeting exons 52 and 53.

AO	length (bp)	Annealing coordinates	exon number	sequences	GC content	
					(bp)	%
1	25	M52A+17+41	52	5' ucc aau ugg ggg cgu cuc ugu ucc a 3'	14	56
2	30	M52A-15+15	52	5' auc uug cag ugu ugc cug aaa gaa aaa aaa 3'	10	33
3	30	M52D+15-15	52	5' cau uaa gag acu uac uuc gau cag uaa uga 3'	10	33
4	30	M52A+22+51	52	5' aau gag uuc uuc caa uug ggg gcg ucu cug 3'	15	50
5	30	M52A+32+61	52	5' ggg cag cag uaa uga guu cuu cca auu ggg 3'	15	50
6	30	M52A+42+71	52	5' uuc aaa uuc ugg gca gca gua aug agu ucu 3'	12	40
7	31	M53A+39+69	53	5' cau uca acu guu Guc ucc ugu ucu gca gcu g 3'	15	48
8	30	M53A-15+15	53	5' ucu gaa uuc uuu caa cug gaa uaa aaa uaa 3'	7	23
9	30	M53D+15-15	53	5' aug cuu gac acu aac cuu ggu uuc ugu gau 3'	12	40
10	30	M53A+29+58	53	5' ugu cuc cug uuc ugc agc ugu ucu uga acc 3'	15	50
11	30	M53A+49+78	53	5' uua aca uuu cau uca acu guu guc ucc ugu 3'	10	33
12	30	M53A+59+88	53	5' guu gaa ucc uuu aac auu uca uuc aac ugu 3'	9	30
13	30	M53A+69+98	53	5' cag cca uug ugu uga auc cuu uaa cau uuc 3'	11	37
14	30	M53A+19+48	53	5' ucu gca gcu guu cuu gaa ccu cau ccc acu 3'	15	50
15	30	M53A-25+5	53	5' uuc aac ugg aau aaa aau aag aau aaa gaa 3'	6	20
16	30	M53A+129+158	53	5' cca uga guc aag cuu gcc ucu gac cug ucc 3'	17	57
17	30	M53A+151+180	53	5' cua cug ugu gag gac cuu cuu ucc aug agu 3'	14	47
18	30	M53A+176+205	53	5' ucu gug auc uuc uuu ugg auu gca ucu acu 3'	11	37
19	30	M53D+5-25	53	5' uuu uaa aga uau gcu uga cac uaa ccu ugg 3'	10	33
20	25	M53A-25-1	53	5' cug gaa uaa aaa uaa gaa uaa aga a 3'	5	20
21	25	M53A-20+5	53	5' uuc aac ugg aau aaa aau aag aau a 3'	5	20
22	25	M53A+69+93	53	5' auu gug uug aau ccu uua aca uuu c 3'	7	28
23	25	M53A+74+98	53	5' cag cca uug ugu uga auc cuu uaa c 3'	10	40
24	25	M53A+99+123	53	5' ccu guu cgg cuu cuu ccu uag cuu c 3'	13	52

Table 2.1 : 2OMe cocktail composition for *in vitro* studies.

cocktail 2OMe AO	A1 1:3	A2 1.66:1	A3 3.33:1	A4 6.66:1	A5 12.33:1
4	150	375	460	520	555
8	150	75	46	26	15
9	150	75	46	26	15
13	150	75	46	26	15
total concentration	600 (nM)	600 (nM)	600 (nM)	600 (nM)	600 (nM)

Table 2.2 : PMO cocktails composition for *in vitro* studies.

cocktail P007-PMO	B1 1:3	B2 1.66:1	B3 3.33:1	B4 6.66:1	B5 12.33:1
4	2.5	6.25	7.7	8.8	9.25
8	2.5	1.25	0.77	0.44	0.25
9	2.5	1.25	0.77	0.44	0.25
13	2.5	1.25	0.77	0.44	0.25
total concentration	10 (μM)	10 (μM)	10 (μM)	10 (μM)	10 (μM)

Table 2.3: PMO cocktails composition for *in vivo* studies.

cocktail P007-PMO	C1 1:3	C2 1.66:1	C3 3.33:1	C4 6.66:1	C5 12.33:1
4	10	25	30	35	37
8	10	5	3.3	1.8	1
9	10	5	3.3	1.8	1
13	10	5	3.3	1.8	1
total amount	40 (μg)	40 (μg)	40 (μg)	40 (μg)	40 (μg)

Table 3 :Primers used for RNA studies and sequencing.

Primers for primary PCR	Sequences (5'-->3')
13Fa	TGC TTC AAG AAG ATC TAG AAC AGG AGC
27Ra	CTA TTT ACA GTC TCA GTA AGG
13Fb	CTC GCT CAC TCA CAT GGT AGT AGT G
26Rb	CCT GCC TTT AAG GCT TCC TT
20Fa	CAG AAT TCT GCC AAT TGC TGA G
35Ra	GGT GAC AGC TAT CCA GTT ACT GTT CAG
20Fb	CCC AGT CTA CCA CCC TAT CAG AGC
35Rb	GCC CAA CAC CAT TTT CAA AGA CTC TCC
37Fa	CCA AAG GTG GAG TCC ACA CGT G
50Ra	CCA GTC GTG CTC AGT CCA GGG
37Fb	GAG CCC CAA ATG TCT GAG CTC
50Rb	GGT TTA CAG CCT CCC ACT CAG
48Fa	GAT TTT AGG CAG CTT GAA GAG
58Ra	TTC CAA AGT GCT GAG CTT ATA AG
48Fb	CTT CTG TGG CTC TCT CCT ATT AG
58Rb	CTC CTG GTA GAG TTT CTC TAG
58Fa	GGG CCT TCA AGA GGG AAT TG
70Ra	CCT CTA ACA CAG TCT GCA CTG G
58Fb	CTA AAG AAC CTG TAA TCA TG
70Rb	GCG AAG TCG CGA ACA TCT TCT CCG
50Fa	GTT AGA AGA TCT GAG GTC TGA GTG
55Ra	GAG TCT TCT AGG AGC TTC TCC TTA
50Fb	CTG TAA ACC ATT TAC TTC GGG AGC
55Rb	CTG TAG GAC ATT GGC AGT TGT TTC

CHAPTER 4

Enhanced exon skipping in the 4^{CV} dystrophic mouse model of muscular dystrophy through refined oligomer design

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4.1 Abstract

Antisense oligomer (AO) induced dual skipping of the dystrophin gene was studied in the B6Ros.Cg-Dmd$^{mdx\text{-}4Cv}$/J (4^{CV}) mouse, an established mouse model of muscular dystrophy. We previously reported by-passing the protein-truncating mutation in exon 53 in this mouse by AO-induced skipping of exons 52 and 53. However, differences in the efficiency of removal of exons 52 and 53 led to a mixture of transcripts, with some missing only exon 53 and others missing both exons. We have developed different AO preparations and report a more efficient combination of AOs that induced almost exclusive expression of the desired transcript missing both exons, first *in vitro* and then *in vivo*. Both intramuscular and intraperitoneal administration of an AO-cocktail preferentially induced the in-frame dystrophin transcript missing both exons and partially restored dystrophin protein expression *in vivo*. The oligomer cocktail also restored assembly of the dystrophin-associated protein complex (DAPC) demonstrated by expression and localisation of DAPC component proteins, and showed some evidence of improving muscle functionality.

4.2 Introduction

Duchenne muscular dystrophy (DMD) is the most common and serious neuromuscular disorder in young adults, affecting about one in 3,500 newborn males. In DMD, the ablation of functional dystrophin leads to loss of the dystrophin-associated protein complex (DAPC), a complex linking the actin cytoskeleton to extracellular matrix, thereby rendering muscle more susceptible to membrane damage. Proximal muscle weakness becomes evident when muscle regeneration is overwhelmed by increased rates of muscle degeneration. DMD patients commonly suffer from cardiovascular and respiratory failure in their late twenties (for review see[1-3]).

Most DMD mutations result in disruption of the reading frame of the dystrophin transcript, thus leading to prematurely truncated non-functional dystrophin.[4, 5] Becker muscular dystrophy (BMD) also arises from mutations in the dystrophin gene, but these defects do not lead to disruption of the reading frame, thereby allowing production of some partially functional dystrophin. BMD phenotypes depend upon the size and position of the mutation, and the clinical presentation can vary from severe (borderline DMD) to asymptomatic.[6-10]

Exon skipping induced by antisense oligomers (AOs) is being investigated as a mechanism to restore dystrophin protein expression in both DMD patients and animal models of muscular dystrophy.[11-17] The B6Ros.Cg-Dmd$^{mdx-4Cv}$/J (4^{CV}) mouse, a model of muscular dystrophy, carries a nonsense mutation in dystrophin exon 53.[18, 19] Removal of both exons 52 and 53 is required to address this protein-truncating mutation, maintain the reading frame and induce the expression of a functional dystrophin isoform. We previously reported that a cocktail of four AOs was capable of removing exons 52 and 53 from the mouse dystrophin transcript and restoring dystrophin protein expression after a single intramuscular injection in the 4^{CV} mouse.[20] However, exons 52 and 53 were excised at different efficiencies by this AO-cocktail, which, in turn, resulted in a predominance of transcripts missing exon 53, compared to the desired in-frame transcript missing both exons 52 and 53. As a consequence, dystrophin expression was less than 10% of that in the control C57BL/10ScSn mouse.

We report further oligomer optimisation experiments to remove dystrophin exons 52 and 53 and compare the efficiency of exon removal with that previously reported. Initial oligomer optimisation was carried out with 2'-O-methyl (2OMe)

phosphorothioate compounds, and subsequently, phosphorodiamidate morpholino oligomers (PMOs) conjugated to a cell-penetrating peptide (P007) were synthesised and administered to 4^{CV} mice, to assess the ability of AOs to increase dystrophin expression and muscle functionality *in vivo.*

4.3 Results

4.3.1 Optimising AO-induced skipping of the individual dystrophin exons 52 and 53

Based on our previous study,[20] a panel of 2OMeAOs, to anneal to splice motifs of exons 52 and 53, including the acceptor and donor splice sites, and potential exonic splicing enhancers (ESEs) of both exons 52 and 53, were selected for further study. **Figure 1** shows the relative annealing positions of the oligomers under investigation.

For exon 52, a total of six oligomers annealing to the acceptor (AO 2), donor (AO 3) and ESEs (AOs 1, 4, 5 and 6) were evaluated. For exon 53, eleven out of eighteen oligomers were selected on the grounds that they annealed to the acceptor (AOs 8, 14, 16 and 17), donor (AOs 9 and 15) and ESEs (AOs 7, 10, 11, 12 and 13).[20] The AOs selected for annealing to ESEs were those that bind to regions +17 to +51 and +29 to +98 on exons 52 and 53, respectively, and which have been shown to individually induce exon skipping.[20] The AOs were combined into five cocktails for exon 52 and fourteen combinations for exon 53 (**Table 1**), and exon skipping efficiencies were estimated after transfection into cultured 4^{CV} myoblasts.

For exon 52, combining AOs annealing to ESEs (AOs 1, 4, 5 or 6) with an AO binding to the acceptor splicing motif (AO 2) and an AO masking the donor site (AO 3) resulted in no improvement in the level of exon skipping, compared to treatment with AO 4 alone, an AO previously shown to be the most effective individual AO[20] (data not shown). However, treatment with the cocktail of AOs 1+6, which combines two non-overlapping AOs, was found to substantially increase the efficiency of exon 52 skipping in 4^{CV} myoblasts, compared to AO 4 (**Fig 2a, b**). The efficiency was emphasised by the dramatic reduction in the amount of full-length gene transcript.

For exon 53, AOs annealing to the mouse dystrophin pre-mRNA acceptor (AOs 8, 14, 16, or 17) and donor splice sites (AOs 9 or 15) were combined with five ESE-masking oligomers (AOs 7, 10, 11, 12, or 13) in fourteen different combinations. The

combination of AOs 8+13+9 was originally identified as the most efficient cocktail.[20] A cocktail of AOs 14+13+9 did not lead to any substantial improvement, but a combination of AOs 8+13+15 was found to enhance levels of exon skipping (**Table 1**).

Subsequently, two combinations comprising AOs masking acceptor and donor splice sites ((AOs 14+9) and (AOs 8+15)) were combined with ESE-annealing AOs (AOs 7, 10, 11, 12 or 13) giving ten different combinations. A cocktail of AOs 8+13+15 was the most effective, compared to the other nine combinations (**Table 1**). Finally, interchanging of different AOs masking the acceptor splice site (AOs 16 or 17) led to no remarkable increase in the efficiency of exon skipping (**Table 1 and Fig 2c, d**). However, there was a greater consistency in the ability of AOs 13+15 to induce robust exon 53 skipping. For this reason, this AO combination was used in subsequent experiments.

4.3.2 Optimising AO-induced dual exon skipping of dystrophin exons 52 and 53

We then hypothesised that a 2OMeAO-cocktail combining AOs with proven efficiency in causing *in vitro* skipping of exon 52 (AOs 1+6), and exon 53 (AOs 13+15), would be effective *in vivo* in inducing dual exon skipping. As the previous study suggested, combining AOs targeting 2 exons at different molar ratio could enhance levels of the desired transcript,[20] hence this cocktail was evaluated at two different ratios (1:1 and 3:1 (referred to as AO-β and AO-δ)) and compared with AOs 4+8+9+13 (AO-α), the AO cocktail described previously (**Table 2**).[20]

Both AO-β and AO-δ were capable of inducing predominant expression of the in-frame dystrophin transcript missing exons 52+53 (Δ52+53), but whilst AO-α induced the Δ52+53 transcript, it also generated a substantial proportion of Δ53 transcripts in four out of five 4^{CV} myoblast cultures (**Fig 2e-g**). There were dystrophin transcripts missing exon 52 in AO-β (**Fig 2f**), and AO-δ-treated 4^{CV} myoblasts, but the levels were lower in AO-δ-treated-myoblasts (**Fig 2g**).

4.3.3 *In vivo* efficacy of AO cocktails in inducing exon skipping after intramuscular PMO administration

AO-α, AO-β and AO-δ were synthesized as peptide-conjugated PMOs, and classified as PMO-α, PMO-β and PMO-δ (**Table 2**). Exon skipping efficiencies were

compared *in vivo* by intramuscular injection of 40 μg of each cocktail directly into *tibialis anterior* (TA) muscles of 4^{CV} mice. Two weeks after treatment, the Δ52+53 transcript was induced at high levels, compared to other dystrophin transcripts (viz. full-length, Δ52, Δ53) in TAs treated with PMO-β and PMO-δ, while levels of the Δ53 transcript were more pronounced in PMO-α-treated muscle (**Fig 3a**). In addition, significant levels of the Δ52+53 transcript were expressed at 4, 6 and 8 weeks after intramuscular administration of PMO-β and PMO-δ, and at two different concentrations (20 and 40 μg). The failure to generate a Δ52+53 product at the 6 week time point (40 μg) is likely to be due to sampling error (**Fig 3b**).

Western blotting was used to confirm dystrophin expression. At equivalent protein loading, dystrophin was barely detectable after 2weeks in PMO-α-treated 4^{CV} mouse TAs, compared to the C57BL/10ScSn muscle sample (**Fig 3c**). However, dystrophin was readily detected within 2 weeks after intramuscular administration of PMO-β or PMO-δ into treated animals. PMO-δ treatment at both 20 and 40 μg restored dystrophin protein to approximately 40% of levels in control C57BL/10ScSn mice at 4 weeks, although this reduced to 7% to 10% at 6 and 8 weeks after treatment (**Fig 3d, e**). Only at a dose of 40 μg was PMO-β found to lead to significant levels of dystrophin protein (**Fig 3e**).

4.3.4 *In vivo* efficacy of AO cocktails in inducing exon skipping after intraperitoneal PMO administration

Intraperitoneal administration of PMO-β and PMO-δ at a dosage of 40 mg/kg twice weekly for 4 weeks, and weekly thereafter until 9 weeks, led to 100% of the induced Δ52+53 transcript in diaphragm of 4^{CV} mice, as determined by RT-PCR. Similarly, RT-PCR analysis of RNA from TA muscles from PMO-β treated mice at 4 and 8 weeks, and PMO-δ-treated mice at 8 weeks showed 100% expression of the Δ52+53 transcript (**Fig 4a**). No Δ52+53 transcript, however, was evident in cardiac muscle from mice treated with PMO-β or PMO-δ, irrespective of the time after PMO treatment (**Fig 4a**).

Western blot analyses of tissues from treated mice corroborated the results of RT-PCR analysis, confirming the expression of dystrophin in diaphragm from PMO-β and PMO-δ treated mice at 4 and 8 weeks (**Fig 4b**). Dystrophin immunostaining of

muscle cryosections from PMO-β and PMO-δ treated mice again showed a high degree of consistency in the expression of dystrophin in diaphragm. However, in TA expression of dystrophin was less pronounced at 4 weeks in PMO-δ treated mice (**Fig 5a**).

4.3.5 *In vivo* efficacy of AO cocktails to restore DAPC and improve muscle functionality after intraperitoneal PMO administration

The observation that PMO treatment led to re-establishment of dystrophin expression in 4^{CV} mice raised the question of whether PMO treatment could restore the DAPC and lead to improved muscle functionality. Preliminary studies using three PMO-δ treated mice sacrificed 8 weeks after a course of intraperitoneal injections, showed that PMO treatment restored the DAPC components, nNOS and β-dystroglycan, to levels comparable to those in the C57BL/10ScSn control mouse, in both diaphragm and TA, albeit to a greater degree in diaphragm (**Fig 5b, c**).

PMO-δ treatment also led to some restoration of functionality in diaphragm (**Fig 6a**), but not extensor digitorum longus (EDL) (**Fig 6b**), based on the extent (%) of damage following an eccentric contraction (5 stretches at 10% optimum muscle length) (**Fig 6a**). Other measures of functionality (viz. twitch force after fatigue, tetanic force and fatigue recovery time), however, showed no significant restoration after PMO treatment (**Fig 6b, c**).

4.4 Discussion

As in our previous study of AO-induced dual exon skipping, we explored the use of oligomer cocktails to excise exons 52 and 53 from the dystrophin gene transcript in the 4^{CV} mouse model of muscular dystrophy.[20] We showed that combining selected AOs that when used individually were inefficient, could induce robust exon skipping. However, not all AO combinations resulted in enhanced exon skipping.

For exon 52, changing the components of the AO cocktails to include AOs annealing to both acceptor and donor splicing motifs resulted in no great improvement in exon skipping efficiency. However, individual AOs masking ESEs (SF2/ASF (IgM-BRCA1), SC35, SRp40 and SRp55) (AO 6) induced inconsistent patterns of exon 52 skipping,[20] but using this AO together with a non-overlapping AO annealing to ESEs (SC35, SF2/ASF and SF2/ASF (IgM-BRCA1)) (AO 1), increased the efficiency of exon

52 skipping in 4^{CV} myoblasts.

Excision of exon 53 also required an AO-cocktail. A 3-AO cocktail (AOs 8+13+9) comprising AOs targeting acceptor and donor splice sites and ESEs, was originally identified as the most active preparation.[20] Substitution of different AOs annealing to the donor splicing motif led to greater levels of exon skipping, whereas changing the composition of AO cocktails to mask the acceptor splice site caused no obvious improvement in AO-induced exon skipping. AO 13 annealing to ESEs (SC35 and SRp40), as in our previous study, appeared to be the best of the ESE-masking AOs when combined in a cocktail, compared to other ESE-annealing AOs. Although masking both obvious splice site and ESEs did not enhance efficiency of exon 52 removal, optimising the annealing coordinates of the donor splice site AO seemed to improve the efficiency of exon 53 skipping.[20] The most effective AO-cocktails targeting exon 53 all included AOs 13+15, subsequently these two compounds were evaluated and found to be of similar efficiency to the 3-AO cocktail in inducing exon skipping. Consequently, this 2-AO cocktail was selected for further studies.

AOs with proven efficiency in inducing *in vitro* skipping of exon 52 (AOs 1+6) and exon 53 (AOs 13+15) were combined at two different molar ratios (1:1 and 3:1 (referred to as AO-β and AO-δ)) because of the relative differences in exons 52 and 53 removal, demonstrated by intermediate products. These cocktails induced expression of the in-frame dystrophin transcript missing exons 52+53 (Δexons 52+53) as the predominant transcript in cultured 4^{CV} myoblasts.

These *in vitro* studies on cultured 4^{CV} mouse myoblasts provided the basis for subsequent *in vivo* studies using either intramuscular or intraperitoneal administration of peptide-conjugated PMOs, PMO-β and PMO-δ. The efficacy of induced dual exon skipping by these PMO cocktails was compared with that of the cocktail (PMO-α) reported in our previous study.[20]

A single intramuscular injection of PMO-β or PMO-δ directly into the TA of 4^{CV} mice resulted in very efficient dual exon skipping, with the Δ52+53 transcript observed two weeks after PMO administration. There was significant expression of the Δ52+53 transcript at 4, 6 and 8 weeks, peaking at 4 weeks after PMO-β and PMO-δ administration, and thereafter declining to approximately 10% by 6 and 8 weeks. Western blotting estimated that PMO-δ, and to a lesser extent PMO-β, restored

dystrophin protein levels to approximately 40% of that in control C57BL/10ScSn mice. Similarly, intraperitoneal administration of PMO-β or PMO-δ for 4 and 8 weeks led to 100% expression (relative to total dystrophin transcript product) of the Δ52+53 transcript in diaphragm and to lesser extent in TA from 4^{CV} mice, as determined by RT-PCR. The expression of the Δ52+53 transcript in PMO-β and PMO-δ treated mice was accompanied by expression of the dystrophin protein in diaphragm muscle and at a lesser extent in TA muscle, as determined by western blotting and immunofluorescence using anti-dystrophin antibody.

No exon skipping effect was observed in cardiac tissue following PMO-β or PMO-δ treatment; we now know that this is due to the nature of the P007 peptide tag conjugated to the PMOs. No induced dystrophin was detected in cardiac tissues after treatment of another PMO with the same P007 peptide tag masking donor splice site of mouse dystrophin exon 23 in *mdx* mice.[12, 13] Most recently, it has been shown that the PMO M23D(+7-18) conjugated to peptide B was able to induce robust dystrophin expression in cardiac tissue.[21-23]

The finding that PMO-β or PMO-δ treatment led to predominant induction of the Δ52+53 transcript and dystrophin protein expression in the 4^{CV} mice prompted us to confirm that these dystrophin changes at the molecular levels led to restored assembly of components of the DAPC and, ultimately, to improved muscle functionality. One of the key roles of dystrophin is to stabilise DAPC, which plays a pivotal role in muscle by linking the cytoskeleton to extracellular matrices[1, 24]. The DAPC also mediates intracellular signals via nNOS and other components[25, 26]. Our preliminary studies confirmed assembly of the DAPC and improved muscle functionality, and are highly encouraging. Levels of the DAPC components, nNOS and β-dystroglycan, were comparable to those in the C57BL/10ScSn control mouse. Moreover, PMO-δ treatment was shown to also lead to some improvement in the extent of damage following eccentric contraction in the diaphragm, but not extensor digitorum longus (EDL) muscles. The limited EDL response, based on the extent (%) of damage following an eccentric contraction, is most probably due to insufficient restoration of dystrophin expression in this muscle. Other measures of functionality (viz. twitch force after fatigue, tetanic force and fatigue recovery time) showed no significant restoration after PMO treatment, which demands that further studies be carried out.

The implications of the these studies for the management of muscular dystrophy in humans are potentially far reaching, given that they confirm that PMO-induced dual exon skipping can lead to substantial dystrophin protein expression, restored assembly of DAPC, and based on preliminary studies, some restoration of muscle functionality.

4.5 Materials and methods

4.5.1 AOs and primers

The sequences of the AOs employed in this study were as reported by Mitrpant *et al.*, 2008.[20] The nomenclature adopted for oligomers is based on that described by Mann et al., 2002.[27] 2OMeAOs were synthesised on an Expedite 8909 Nucleic Acid Synthesiser (Applied Biosystem, Forster, CA, USA.), and PMOs conjugated to an arginine-rich, cell-penetrating peptide (P007)[28, 29] were synthesised by AVI BioPharma (Corvallis, OR, USA). Primers for RT-PCR and sequencing analysis were synthesised by Geneworks (Adelaide, Australia) and are reported elsewhere.[20]

4.5.2 Animals

4^{CV} (B6Ros.Cg-$Dmd^{mdx\text{-}4Cv}$/J) congenic mice, housed and supplied by the Animal Resources Centre, Murdoch University, Western Australia, were used for *in vivo* studies. Procedures for animal housing and transport complied with the guidelines of the National Health and Medical Research Council (Australia). Experimental protocols using animals were approved by the Animal Ethics Committee of the University of Western Australia (Approval Number 03/100/572).

4.5.3 Cell culture and AO transfection

Primary myoblast cultures of 4^{CV} mice were prepared from 2-4 day-old pups by the protocol described elsewhere.[20, 30] 2OMeAOs complexed on a 1:2 basis (w/w) with Lipofectin (Invitrogen, VIC, Australia) were incubated with 4^{CV} myoblasts, as reported by Mitrpant *et al.* 2008.[20] Transfected myoblasts were incubated for 48 hours before RNA was extracted for analysis.

4.5.4 Intramuscular administration

PMOs, prepared in physiological 0.9% (w/v) saline, were injected into mouse TA muscles *in vivo* at the doses indicated in the text. Each experiment included at least one saline-only treated mouse as a negative control. TAs were removed from animals anaesthetised and sacrificed by cervical dislocation at the indicated time points after injection, and snap-frozen in pre-cooled isopentane. TAs were then cryosectioned and prepared for RNA and protein analysis.

4.5.5 Intraperitoneal administration

PMO cocktails, prepared in 50 µl of 0.9% (w/v) normal saline, were injected into the peritoneal cavity of mice at day 7 after birth. PMO cocktails were administered twice a week for 4 weeks, and once a week from weeks 6 to 9. Animals administered saline alone served as controls. PMO-treated and saline-injected animals were anesthetised and sacrificed by cervical dislocation at the indicated time points after injection. Muscles and other tissues were removed, snap-frozen in pre-cooled isopentane. Tissues were then cryosectioned and prepared for RNA and protein analysis.

4.5.6 RNA extraction, RT-PCR analysis, and DNA sequencing

RNA was harvested from 4^{CV} cultural myoblasts and tissue cryosections using Trizol (Invitrogen), according to the manufacturer's protocol. One-step RT-PCR followed by nested PCR was undertaken according to protocols described elsewhere.[20] PCR products were separated on 2% agarose gels in TAE buffer, pH 8.3 and images captured on a Vilber Lourmat CHEMISMART-3000 gel documentation system (Vilber Lourmat, Marne-la-vallee, France). PCR products were re-amplified and identified by DNA sequencing following protocols reported elsewhere.[31]

4.5.7 Western blot analysis

Mouse muscle cryosections were homogenised in treatment buffer (4.5 mg tissue per 100 µl buffer) as described in Mitrpant *et al.*, 2008.[20] Protein extracts were fractionated by electophoresis on a 4-10% SDS gradient gel at pH 8.8 with a 4% stacking gel, pH 6.8. Densitometry of myosin bands after coomassie blue staining was undertaken to ensure equivalent levels of protein loading. Proteins were transferred

from the gel to nitrocellulose membranes (Amersham Biosciences, Castle Hill, Australia) overnight at 18°C, at 290mA. Dystrophin was visualised using NCL-DYS2 monoclonal anti-dystrophin antibody (Novocastra, Newcastle-upon-Tyne, UK) as described previously.[20] Images were captured on a Vilber Lourmat Chemi-Smart 3000 gel documentation system (Vilber Lourmat).

4.5.8 Tissue preparation and immunofluorescence

Dystrophin was detected on unfixed cryosections using NCL-DYS2, an antibody that reacts strongly with the C-terminus of dystrophin. Immunofluorescence was performed using a Zenon Alexa Fluor 488 labelling kit (Invitrogen), according to the protocol recommended by manufacturer, except that fixation was delayed until after staining. The primary antibody was diluted 1:10 and sections were counterstained with Hoechst (Sigma-Aldrich, St. Louise, MO, USA) at a dilution of 1:10,000 to visualise nuclei. Sections were viewed under an Olympus IX 70 inverted microscope and images captured on an Olympus DP70 digital camera.

4.5.9 Functional testing of isolated diaphragm strips and EDLs

The entire diaphragm muscle and surrounding ribcage, were excised and placed in a dish containing oxygenated physiological saline solution containing 10mM sodium pyruvate and 1% foetal bovine serum. Diaphragm strips and extensor digitorum longus (EDLs) were dissected and tied with surgical silk at the central tendon and at the distal end. Diaphragm strips and EDLs were then transferred to a muscle test chamber containing physiological saline maintained at 25°C continuously bubbled with 95% O_2, 5% CO_2. The contractile properties of the muscles were evaluated using an *in vitro* muscle test system (Aurora Scientific Inc., Milford, PA, USA) to determine the maximum isometric twitch force (Pt), maximum isometric tetanic force (Po) and the frequency force relationship (20 to 150 Hz). After determination of their isometric contractile properties, muscles were subjected to an eccentric contraction protocol to determine susceptibility to stretch-induced muscle damage. After functional testing, the muscle mass, fibre length, and Po were recorded to calculate the specific force relative to muscle fibre cross-sectional area (kN/m^2).

4.6 References

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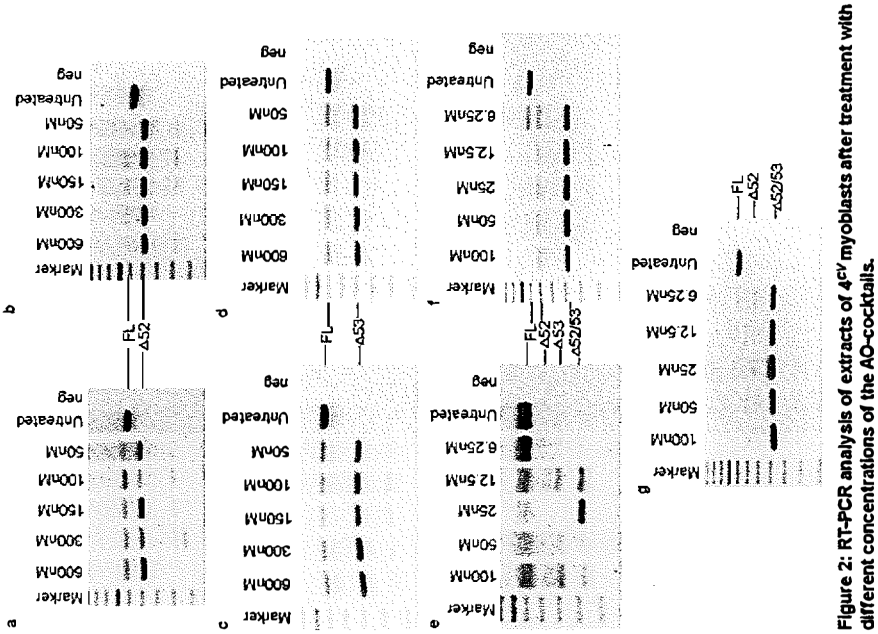


Figure 2: RT-PCR analysis of extracts of 4^{cv} myoblasts after treatment with different concentrations of the AO-cocktails.

(a) AO 4; (b) AOs 1+6; (c) AOs 8+13+9; (d) AOs 13+15; (e) 2OMeAO-α (AOs 4+8+9+13); (f) 2OMeAO-β (AOs 1+6+13+15); and (g) 2OMeAO-δ (AOs 1+6+13+15) compared to untreated 4^{cv} myoblasts and saline-only negative controls. The full-length transcript (FL) are shown as well as transcripts missing exon 52 (Δ52), exon 53 (Δ53), and both exons 52 and 53 (Δ52/53). The size of transcripts was determined by comparison to molecular weight markers and confirmed by DNA sequencing.

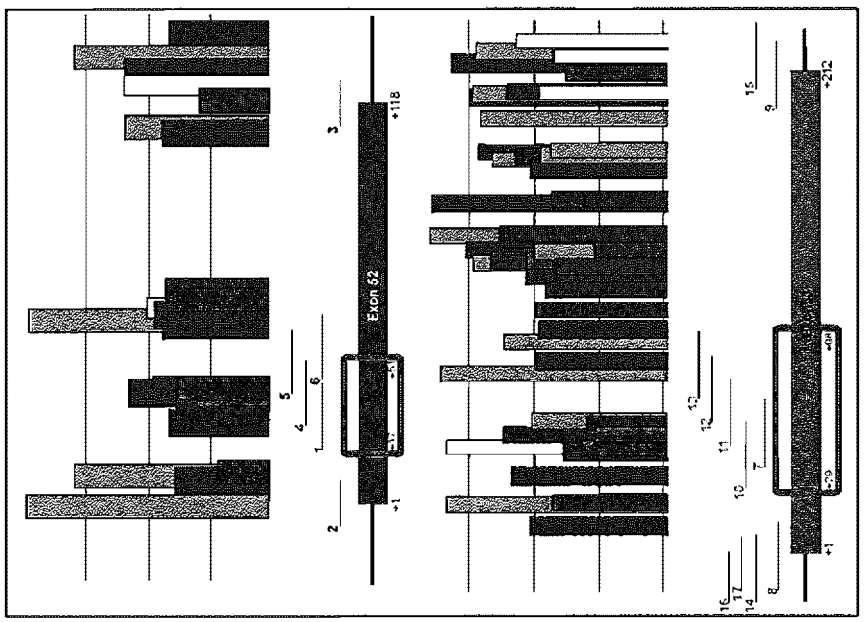


Figure 1: Annealing positions of selected AOs targeting exons 52 and 53.

Diagrammatic representation of the predicted ESEs in exons 52 and 53 and the relative annealing coordinates of the selected AOs. Vertical bars indicate the five ESEs predicted to be binding sites of SR proteins (viz. SF2/ASF (red), SF2/ASF (IgM-BRCA1) (pink), SC35 (navy blue), SRp40 (light green) and SRp55 (yellow)). Threshold scores were set at default values as specified in ESE finder.[32, 33]

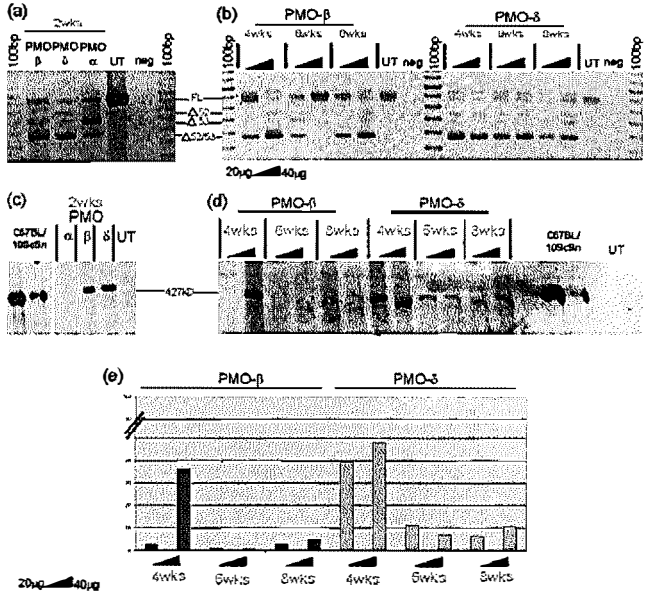


Figure 3: (a and b) RT-PCR. The full-length transcript (FL) transcripts missing exon 52 (Δ52), exon 53 (Δ53), and both exons 52 and 53 (Δ52/53) are indicated.

The size of transcripts was determined by comparison to molecular weight markers; (c and d) Western blot analysis. Semi-quantification of restored dystrophin was determined by densitometric analysis of western blots of samples from treated mice relative (%) to dystrophin protein levels in C57BL/10ScSn control mice; and (e) semi-quantification of restored dystophin protein in TAs of the 4 mice which were intramuscularly injected with PMO-α, PMO-β and PMO-δ.

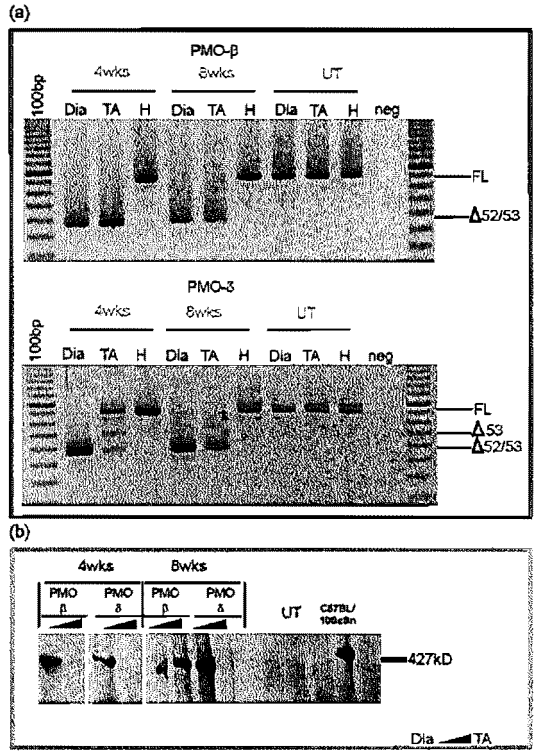


Figure 4: Levels of (a) dystrophin RNA and (b) protein in diaphragm and TA muscles derived from the 4^{cv} mice intraperitoneally injected with PMO-β and PMO-δ as determined by RT-PCR and western blot analysis, respectively.

The levels of full-length transcript (FL) are shown as well as levels of the transcripts missing exon 52 (Δ52), exon 53 (Δ53), and both exons 52 and 53 (Δ52/53). The size of transcripts was determined by comparison to molecular weight markers.

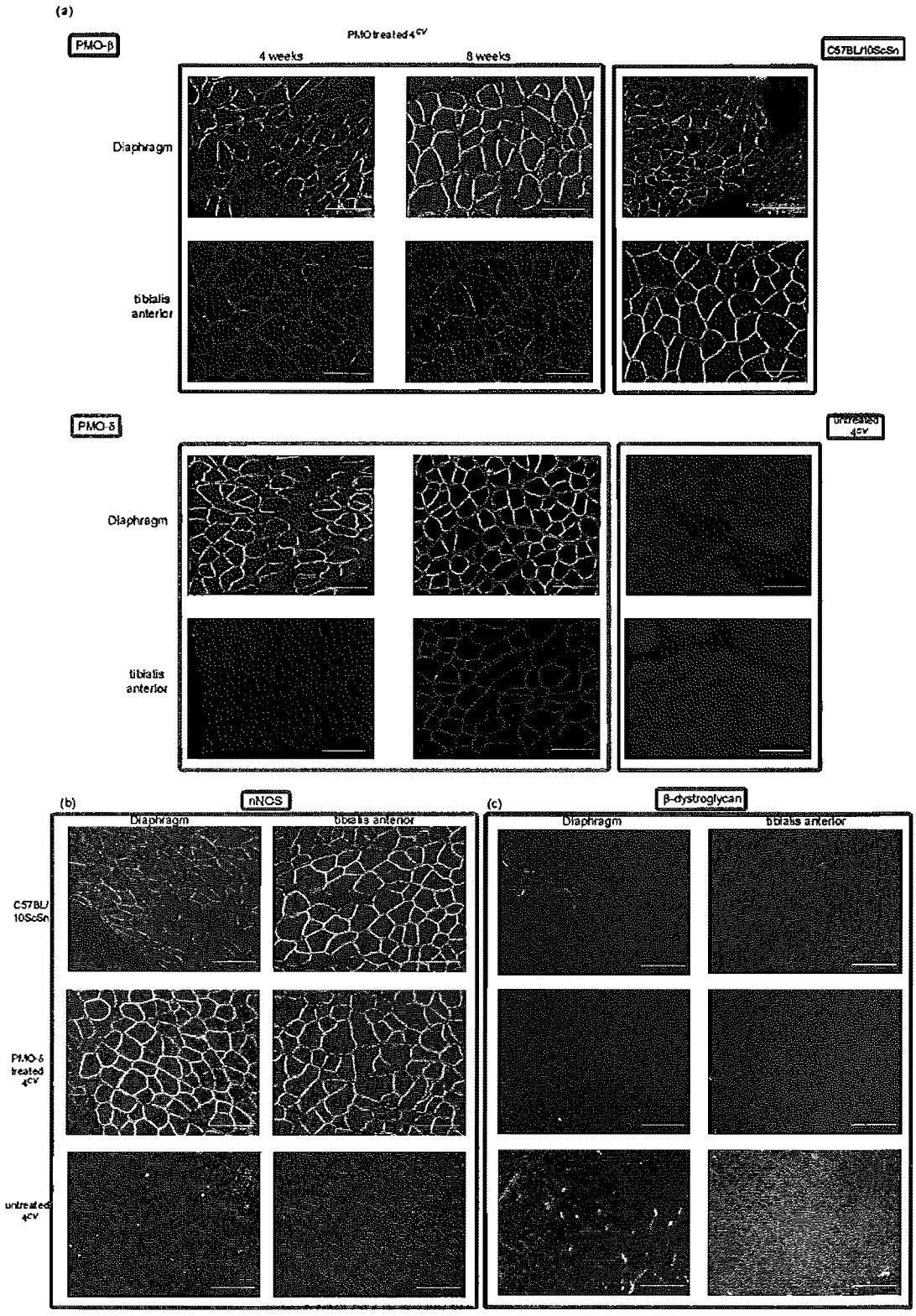
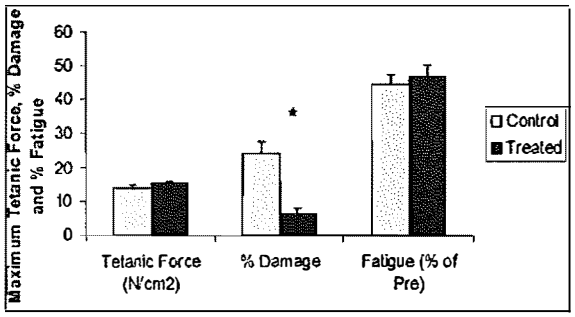


Figure 5: Immunofluorescence of (a) dystrophin, (b) neuronal nitric oxide synthase (nNOS) and (c) β-dystroglycan in diaphragm and TA muscle cryosections from PMO-β and PMO-δ treated 4^{cv} mice.

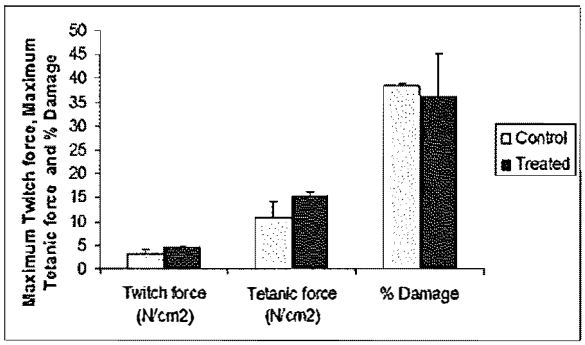
Dystrophin immunofluorescence was examined at 4 and 8 weeks after intraperitoneal PMO injection in 4^{cv} mice and compared to that on cryosections from C57BL/10ScSn and untreated 4^{cv} mice. nNOS and β-dystroglycan immunostaining was examined at 8 weeks after PMO-δ treatment and compared to C57BL/10ScSn and untreated 4^{cv} mice. (bar=100μm).

(a)



* P < 0.05 between control and treated.

(b)



(c)

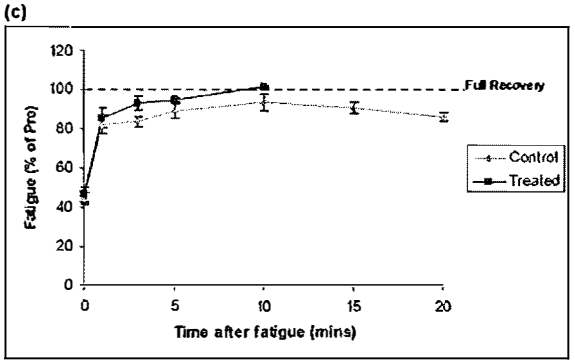


Figure 6: Contractile properties of (a and c) diaphragm; and (b) extensor digitorum longus (EDL) muscles derived from the 4^{CV} mice intrapertoneally treated with PMO-8 for 8 weeks.

Four EDLs and three diaphragms from PMO-8 treated 4^{CV} mice were included, whereas four EDLs and two diaphragms from untreated 4^{CV} mice were used in the study.

Table 1: The composition of the AO-cocktails to remove either exons 52 or 53.

The compositions of the different AO-cocktails tested targeting exons 52 and 53 and their efficiency in causing exon skipping (as scored subjectively on a 4-point scale by visual analysis of >2 separate RT-PCR gels).

compositions of selected cocktails	exon skipping efficiency
AOs 1+8	+++
AOs 2+1+3	++
AOs 2+4+3	++
AOs 2+5+3	+
AOs 2+6+3	+
AO 4	++
AOs 8+13+9	+++
AOs 8+7+15	++
AOs 8+10+15	++
AOs 8+11+15	+++
AOs 8+12+15	+++
AOs 8+13+15	++++
AOs 14+7+9	++
AOs 14+10+9	++
AOs 14+11+9	+++
AOs 14+12+9	+++
AOs 14+13+9	+++
AOs 14+13+15	++++
AOs 16+13+15	++++
AOs 17+13+15	++++
AOs 13+15	++++

Table2: The composition of the selected cocktails to remove exons 52+53 and their efficiency in causingexon skipping.

The compositions of the three selected AO-cocktails targeting both exons 52 and 53 and their efficiency in causing skipping of both exons, based on levels of dystrophin protein, determined by densitometric analysis of western blots and expressed (%) relative to dystrophin protein levels in control C57BL/10ScSn mice.

compositions of selected cocktails	molar ratios of AOs targeting exons 52:53	2OMeAO cocktails	PMO cocktails	Percentage of rescued dystrophin protein
AOs 4+8+9+13	1.66:1	2OMeAO-α	PMO-α	7%
AOs 1+6+13+15	1:1	2OMeAO-β	PMO-β	40%
AOs 1+6+13+15	3:1	2OMeAO-δ	PMO-δ	40%

CHAPTER 5

Rational design of antisense oligomers to induce dystrophin exon skipping

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5.1 Abstract

Duchenne muscular dystrophy (DMD), the most severe neuromuscular disorder of childhood, is caused by the absence of a functional dystrophin. Antisense oligomer induced exon skipping is being investigated to restore functional dystrophin expression in models of muscular dystrophy and DMD patients. One of the major challenges will be the development of clinically relevant oligomers and exon skipping strategies to address many different mutations. Various models, including cell-free extracts, cells transfected with artificial constructs, or mice with a human transgene, have been proposed as tools to facilitate oligomer design. Despite strong sequence homology between the human and mouse dystrophin genes, directing an oligomer to the same motifs in both species does not always induce comparable exon skipping. We report substantially different levels of exon skipping induced in normal and dystrophic human myogenic cell lines and propose that animal models or artificial assay systems useful in initial studies may be of limited relevance in designing the most efficient compounds to induce targeted skipping of human dystrophin exons for therapeutic outcomes.

5.2 Introduction

Duchenne muscular dystrophy (DMD), the most severe neuromuscular disorder of childhood, is caused by the absence of a functional dystrophin,[1] with patients suffering from progressive muscle weakness and severe respiratory and cardiac complications by the second and third decades of life.[2, 3] Becker muscular dystrophy (BMD) also arises from mutations in the dystrophin gene, but these lesions are such that some functional protein can be generated, albeit of reduced quantity and/or quality.

Splice intervention using antisense oligomers (AOs) is being developed as a potential molecular therapy for DMD. AO intervention during dystrophin pre-mRNA processing aims to exclude one or more exons associated with the primary DMD-causing mutation, while maintaining or restoring the dystrophin reading frame. A clinical trial of AO induced exon skipping in DMD patients has demonstrated proof of principle that this antisense strategy can restore some dystrophin expression in DMD muscle.[4]

One of the major challenges to oligomer induced gene transcript manipulation for therapeutic purposes, will be the design and development of clinically relevant oligomers. Various models, including cell-free extracts, cells transfected with artificial constructs, mice with a human transgene, and *in silico* predictions, have been proposed as tools to facilitate oligomer design for splice manipulation.[5-7] The highly coordinated nature of gene expression leads to reduced efficiency of processing when cell-free extracts are used to assay splicing. Within a nucleus, introns are removed an estimated forty times faster than *in vitro* processing of synthetic pre-mRNA transcripts.[8] Normal gene expression appears precariously balanced when one considers minor base changes can lead to activation of cryptic splice sites, pseudo-exon inclusion or some other form of aberrant splicing.[9, 10] Hence, insertion of any test exon with some arbitrarily selected flanking intronic sequences into a splice reporter system may allow some assessment of oligomer induced redirection of splicing patterns in transfected cells. However, such a system will not directly reflect processing of that exon when it is under control of the full complement of tissue specific cis- and trans- splicing motifs and factors. Finally, transcription-coupled processing differs from uncoupled processing in that the nascent pre-mRNA is a growing strand that is constantly folding into new structures and associating with protein-RNA and protein-protein complexes within the "mRNA factory".[11] The majority of *in silico* predictions are the result of analysis of imported

sequences of arbitrarily selected length.

We consider normal human myogenic cells appropriate for AO design and optimisation, and reported an initial draft of AOs targeting human dystrophin exons 2 to 78.[12] Despite strong homology between the mouse and human dystrophin genes, we found variation in exon skipping efficiency and patterns of exon removal in these transcripts. For some exons, directing oligomers to the same target motifs induced similar exon skipping patterns. In contrast, oligomers targeting other exons generated different dystrophin splice isoforms in the two species. We report substantially different levels of exon skipping induced in normal and dystrophic human myogenic cell lines, and propose that animal models or artificial assay systems useful in some studies may be of limited relevance in designing the most efficient compounds to induce targeted skipping of human dystrophin exons.

5.3 Results

5.3.1 Splice site and auxiliary motif predictions

Acceptor and donor splice site scores for exons under investigation, as calculated by the web-based program, MaxEntScan are shown in **Table 1**, along with details of exon and flanking intron lengths. Although there was some variation in the mean donor splice site strength of those exons under investigation, there was no obvious trend where strong or weak donor splice sites were found to be more responsive as targets for splice intervention. Similarly, the score for the acceptor splice sites did not offer any indication as to the suitability of the sites for AO induced exon skipping. It is of interest that the mouse dystrophin exon 23 acceptor splice site, predicted to be very weak, has previously been shown to be unresponsive as a target for exon removal.[13]

Exons and limited flanking intronic sequences were analysed for Exonic Splice Enhancers (ESEs), using two programs: ESEfinder 3.0 predicts motifs responsive to the human SR proteins SF2/ASF, SC35, SRp40 or SRp55,[14, 15] and RESCUE-ESE allows identification of putative ESEs for human and mouse.[16, 17] AO sequences designed to excise selected exons, and predicted ESEs, within these annealing sites are described in **Table 2.** The distribution of splice motifs predicted by RESCUE-ESE, relative AO annealing, and induced exon skipping patterns for both human and mouse dystrophin exons under investigation are shown in **Figures 1a-e.** The most effective AOs appear

to preferentially target predicted SF2/ASF (IgM-BRCA1) (25%) or SC35 (28%) motifs, compared to 22%, 17% and 8% targeting SF2/ASF, SRp40, SRp55, respectively.

5.3.2 AO design and evaluation

A direct comparison of relative AO induced skipping of the same exon in immortalized *mdx* mouse and human myogenic cells was undertaken to establish if annealing coordinates for one species could be directly applied to the other. Immortalized *mdx* cells were assessed as removal of exon 23 would by-pass the nonsense mutation, and exon 23 skipped transcripts would no longer be subjected to nonsense mediated decay, unlike the intact, full-length transcript. Removal of other exons from the *mdx* dystrophin gene transcript would not remove the premature stop codon, hence both full length and skipped transcripts should be subjected to equal rates of nonsense mediated decay. Exclusion of selected exons from the normal dystrophin gene transcript will, in many cases, disrupt the reading frame and render the induced transcript subject to nonsense-mediated decay. In most experiments, dose responses of exon skipping with varying oligomer concentration were used to rank oligomer efficiency for that exon. A comprehensive series of AOs has been developed to excise each exon from the human dystrophin gene transcript.[12, 18, 19] Oligomer induced exon skipping of several mouse dystrophin exons has been examined in normal and dystrophic mice, and is an on-going process of refinement.[20, 21]

In three of 10 exons under investigation, optimal annealing co-ordinates for induction of exon skipping were identical in human and mouse, indicating that transfer of AO design between human and mouse dystrophin exons 19, 21 and 25 was possible (data not shown). As shown in **Table 1**, the calculated acceptor splice site scores for exon 19 was high (9.07-9.24), while the donor splice site scores were moderate (5.07-6.49). In contrast, the scores for exon 21 acceptor (4.63-6.14) were much weaker than the donor (9.07-10.08), whereas the acceptor and donor site scores of exon 25 were of intermediate values (4.36-7.46 and 7.25-9.46, respectively).

Exons 19, 21 and 25 were removed by single AOs, as were exons 22 and 23, although in the latter cases there were substantial differences in exon skipping efficiency and splicing patterns between the species. For both mouse exons 22 and 23, the donor splice sites were originally identified as amenable targets for exon excision, however, when these coordinates were targeted in the human dystrophin pre-mRNA,

there was no detectable exon skipping (**Figs 1a, b**). Targeting intra-exonic motifs in human dystrophin exons 22 and 23 induced substantial exon skipping, and directing AOs to these coordinates in the mouse dystrophin pre-mRNA also resulted in efficient exon excision. Indeed, it would appear that directing an oligomer to the corresponding mouse intra-exonic motif identified in human dystrophin exon 22 was equally effective at excising the target exon, as the oligomer annealing to the donor splice site. The induced splicing patterns for exon 23 also differed between human and mouse, in that while human exon 23 removal was specific (1216 bp), transcripts missing exons 22 and 23 (1070 bp) were readily detected in the treated mouse cells, in addition to specific exon 23 skipping. This dual exon skipping was more pronounced when the mouse exon 23 donor site was targeted, and it would appear that directing an oligomer to the intra-exonic motif may result in more specific exon 23 skipping.

Human exon 24 could be excised with a single AO, H24A(+51+73) (1309 bp), but RT-PCR also revealed the presence of transcripts missing exons 24+25 (1087 bp). Targeting the equivalent mouse coordinates resulted in lower levels of exon 24 skipping in an apparent non-dose dependant manner. More efficient and specific mouse exon 24 skipping was induced using a combination of two AOs. The application of an AO cocktail targeting the equivalent coordinates induced skipping of human exon 24, but transcripts missing exon 24 and 25 were still evident (**Fig 1c**).

Two of the 10 human exons (20, 65), and 4 of the 10 mouse exons (20, 24, 52, 53) were only efficiently dislodged by the application of combinations of AOs. There was partial overlap in AO annealing coordinates for the cocktails that excised exon 20 from both the human (H20A(+44+71) and H20A(+147+168)) and mouse ((M20A(+23+47) and M20A(+140+164)) dystrophin gene transcripts (**Table 2**).[12, 21, 22] The human and mouse AO cocktails appeared equally efficient when used in mice, but when the optimal mouse coordinates were directed to the human dystrophin pre-mRNA, there was a slight, but reproducible decline in efficiency of exon removal, most noticeable at lower transfection concentrations (**Fig 1d**).

A single AO, H52A(+12+41) was found to efficiently remove human exon 52 from the mature dystrophin transcript, with greater than 30% exon skipping being induced after transfection at 100 nM.[12] Directing an oligomer to the corresponding mouse coordinates induced substantial exon skipping, but the application of the cocktail M52A(+17+41) and M52A(+42+71) resulted in four-fold more exon exclusion (data

not shown).

Directing AOs to human and mouse dystrophin exons 53 resulted in the generation of the most distinct patterns of exon excision observed to date. A single AO, H53A(+39+69), was able to induce efficient and specific exon skipping from the human dystrophin gene transcript (**Fig 1e**). Upon targeting the same coordinates in the mouse dystrophin pre-mRNA, some transcripts missing exon 53 (700 bp) were detected, as well as a substantial proportion of transcripts missing both exons 53 and 54 (545 bp). The shorter transcript is out-of frame and was never detected after transfecting human cells with this AO, or any of the other 18 AOs designed to excise human exon 53.[20] Specific mouse exon 53 skipping could be induced using a combination of two mouse-specific AOs (**Fig 1e**). However, when the same annealing coordinates were targeted in the human dystrophin gene transcript, only low levels of exon 53 skipping were detected (**Fig 1e**).

Two additional AOs (M53A(+151+180) and M53A(+176+205)), with overlapping annealing sites, were found to induce cryptic splicing in mouse exon 53, which led to the loss of 78 bases of coding sequence from 3′ end of the exon (**Fig 2a**). The activated mouse cryptic donor splice site was identified by DNA sequencing (**Fig 2b**), and calculated to have a splice site score of 7.41/7.26 (**Fig 2c**). Directing oligomers to this region of the human gene transcript resulted in low levels of inconsistent exon 53 skipping, with no evidence of cryptic splice site activation (data not shown).

Human dystrophin exon 65 could only be efficiently removed using a combination of two AOs,[22] whereas a single AO, M65A(-11+14), annealing to the same acceptor splice site coordinates as one of the human cocktail components was able to induce exon skipping from the mouse dystrophin pre-mRNA after transfection at concentrations as low as 50 nM. Another oligomer targeting mouse exon 65, M65A(+63+87) (shown in **Table 2**), was found to induce more robust exon skipping, but like the two compounds in the optimised human exon 65 cocktail, this oligomer did not induce human exon 65 skipping when applied individually (data not shown.)

5.3.3 Induced exon 16 skipping in normal and dystrophic human myogenic cells

Two AOs, H16A(-12+19)[12, 23] and H16A(+11+35)[23], previously designed to excise exon 16 from the mature human dystrophin mRNA, could be readily

distinguished in their ability to excise the target exon (**Figs 3a, b**). H16A(-12+19), targeting the exon 16 acceptor site could induce pronounced exon excision at a concentration of 10 nM, while H16A(+11+35) induced weaker exon 16 skipping after transfection at 25 nM in normal human myogenic cells. However, when these compounds were transfected into myogenic cells from a DMD patient with a G>T substitution of the first base of intron 16 (IVS16+1G>T; c.1992+1G>T), both AOs induced robust exon skipping and could not be readily discriminated (**Figs 3c, d**). RT-PCR on RNA extracted from the untreated DMD cells did not yield a consistent pattern, with sporadic generation of shorter than normal transcripts missing exons 13-15, 14-16, 14+16 (exon 15 present) and 16, and less abundant, near-normal, or larger than expected products. Some of the products of abnormal size were identified by DNA sequencing as having arisen from displacement of the exon 16 donor splice site by one base upstream, or pseudo-exon inclusion of 89 bases from intron 16 (data not shown).

5.4 Discussion

Pre-mRNA splicing, the process of joining exons and removing intervening sequences, is tightly controlled by complex interactions between cis-elements and more than 150 trans-factors[24-27] involved in recognition of exon boundaries and subsequent incorporation into the mature transcript. Four classical cis-elements; the 5′ and 3′ splice site, the polypyrimidine tract, and branch point are primary binding sites for snRNPs and other proteins involved in defining exon/intron boundaries. In addition to the obvious splice motifs, exon recognition and splicing also depends upon the nature, position and combination of auxiliary splice motifs that modulate signals determining exon incorporation, presumably by recruiting trans-factors that regulate exon selection.[28, 29]

The high degree of conservation at the ends of the introns would suggest that the acceptor and donor splice sites are obvious and preferable targets for AO induced exon skipping. Since all dystrophin exons are constitutively expressed in the predominant 427 kDa skeletal muscle isoform, splice site scores are somewhat redundant, but may provide an indication of amenable targets to redirect pre-mRNA splicing. However, considering the 10 exons examined in this report, there was no obvious correlation between predicted splice site score, and optimal AO target. Donor splice sites seemed to be the least preferred human targets, although these motifs were amenable targets in skipping 3 of the 10 mouse exons.

AO-induced exon skipping requires appropriately designed AOs to specifically mask motifs involved in the exon recognition and splicing process, by either rendering the single stranded motifs double-stranded or altering the secondary structures essential for normal exon recognition and processing. Robust and consistent exon skipping is essential if oligomers are to be clinically applicable. If it is necessary to use large amounts of an oligomer to excise a target exon, this may be difficult to achieve in a clinical setting, and/or lead to non-antisense effects. Although 12 oligomers may be capable of restoring the dystrophin reading frame in DMD individuals with exon deletions in the mutation hotspots,[30] it will be essential to extend this therapy to many different amenable mutations across the gene transcript.

Subtle DNA changes in the dystrophin gene (micro-insertion/deletion, nonsense, splice site mutations) represent an estimated 30% of DMD cases. These mutations cannot be ignored based only upon incidence, especially since the gene is largely intact and in most of these cases, splice intervention should result in a dystrophin isoform of near-normal function. Furthermore, protein-truncating mutations in the exons encoding the rod domain could be considered most amenable to exon skipping. The majority of exons in this region are in-frame and hence removal of a nonsense mutation/micro-insertion/deletion would only require excision of a single exon. In-frame deletions within this region are not commonly reported, possibly because many of these cases are not recognised due to a mild phenotype.[31] Indeed, it has been reported that an individual missing exon 16 had no clinical symptoms and normal serum creatine kinase levels, a sensitive marker of muscle damage.[32]

The 45 AOs evaluated for both human and mouse dystrophin pre-mRNA annealed to a total of 174 predicted ESEs. SF2/ASF (IgM-BRCA) and SC35 were found to be more common targets for the optimized AO-induced skipping of these dystrophin exons, consistent with other reports.[22, 33] The high proportion of AOs capable of redirecting splicing supports the concept that many pre-mRNA motifs are involved in exon definition and splicing (for review see[11, 34, 35]). It is possible that the importance of some motifs in pre-mRNA processing is reflected by the levels of induced shortened transcripts. It should also be noted that no single motif is a universal optimal target. Mouse exons 22 and 23 were efficiently excised by targeting two distinct domains, the donor splice sites and intra-exonic motifs, which were identified during a study of the human gene transcript. The human dystrophin gene transcript

only responded to AOs directed at intra-exonic targets for these exons.

Although some AO coordinates appeared to be equally amenable in the two species, there were differences, some subtle and others more pronounced, that must raise questions regarding the accuracy and validity of AO design in non-homologous or artificial systems. Directing an AO at coordinates found to efficiently excise exon 53 from the human dystrophin mRNA, induced dystrophin gene transcripts missing exons 53 and 53+54 when applied to mouse cells. There are several cases where a single AO can excise two exons at a time, presumably reflecting closely coordinated processing.[30, 36, 37] Targeting human exon 53 resulted in only the loss of that exon from the mature dystrophin transcript, but interestingly, directing an AO to human dystrophin exon 54 led to removal of both exons 54+55.[12] An AO designed to excise mouse exon 54 resulted in specific removal of that targeted exon (unpublished data). Although similarities in human and mouse dystrophin splice motif usage were predicted to be as high as 90% in constitutive splicing,[38-40] differences in genetic background and splice motif usage must be considered when extrapolating transcript manipulation from one model to another.

During initial optimisation studies to induce mouse exon 53 skipping, two overlapping AOs were found to activate a cryptic donor splice site in that exon, which led to the loss of 78 bases of coding sequence. The activated mouse exon 53 cryptic splice site was calculated to have a splice site score similar to the wild type donor site. When targeting the same coordinates in the human dystrophin transcript, activation of cryptic splicing was never detected. A 'T' (mouse) and 'C' (human) difference allowed activation of a cryptic donor splice site only in the mouse transcript, thereby demonstrating that species-specific variation, even when not directly affecting AO annealing, can have indirect and unexpected consequences on gene expression.

We consider it important to initially optimize AO design in myogenic cells expressing a normal dystrophin gene transcript, even though protein studies are not possible. Induced exon removal from the intact dystrophin mRNA would ensure that the intervention was possible in the presence of all normal transcription and splicing cis-elements and splicing machinery, thus setting a high standard in AO design. Exonic deletions that disrupt the reading frame and lead to DMD would compromise pre-mRNA processing to some extent, because of the loss of splice motifs in the deleted region. A deletion of exon 50 and flanking intronic sequences will bring together exons 49 and 51 that should not be in direct communication in the context of normal

dystrophin transcript processing. Furthermore, a normal dystrophin gene transcript would not be subjected to nonsense-mediated-decay, unlike induced transcripts in which exon removal causes a frame-shift.[34]

We previously reported optimisation of AOs to excise exon 16 from the human gene transcript.[23] The addition of 5 nucleotides to the AO was found to increase efficiency of target exon skipping by approximately forty-fold, more than justifying a 20% increase in the length of the oligomer. The exon 16 donor splice site mutation did not lead to a single aberrant gene transcript, but a mixture of products, including shorter in-frame transcripts that should have mitigated severity of the disease. Since the diagnosis of DMD had been confirmed clinically, it is most likely that the in-frame products represent very low abundance mRNAs that had escaped NMD and were hence detected by RT-PCR. A nonsense mutation in the muscle glycogen phosphorylase gene was reported to result in 7 different gene transcripts generated by altered splicing.[41] While it appears that the exon 16 dystrophin donor splice site mutation generated multiple disease-associated transcripts, the AO that excised exon 16 restored apparently normal levels of a single in-frame transcript.

The evidence that single base variation between mouse:human or human:human dystrophin genes can influence splice manipulation must cast some doubts on assays that do not assess dystrophin splice manipulation in the appropriate environment. There is no doubt that *in vitro* gene expression differs from that in equivalent cells *in vivo*. However, dystrophin processing in cultured myogenic cells is likely to be more relevant than an artificial system examining transgene expression in a cell line transfected with a plasmid construct containing only a portion of the dystrophin gene taken out of context. Goren *et al.*[40] demonstrated that the same auxiliary splice motif could direct either exon inclusion or exclusion from different mini-genes, depending on the location of splice motifs in the construct. When sequences flanking splice regulatory motifs are manipulated, splicing is modified.[40] In such an artificial system, irrelevant intron size and sequences must be a major concern for AO design and optimization.

In summary, the design and evaluation of AOs to induce human dystrophin exon skipping should be undertaken in human myogenic cultures, whereas a study of exon excision from the mouse dystrophin gene transcript should be undertaken in murine cells. Despite strong homology between the human and mouse (and canine) dystrophin genes, there are many differences and some, as described in this report, influence RNA

processing. When developing exon skipping strategies for non-deletion DMD patients, AOs designed according to the normal dystrophin gene may not be ideal. The disease-causing base change, loss or insertion may occur at the AO annealing site and compromise its ability to excise that exon. Although it is possible that the change may alter a splice motif and enhance exon recognition and strengthen splicing, it is more probable that most changes in a gene would weaken exon recognition and splicing. Regardless of the predicted consequences of a change in dystrophin splicing, testing should be undertaken in myogenic cells, then dystrophic cells carrying the mutation under examination, and ultimately the patient.

5.5 Materials and methods

5.5.1 Splice site scoring and prediction of ESEs motifs

Acceptor and donor splice site sores for all exons under analysis were determined using two different algorithms; the maximum entropy model (MEM) and the First-order Markov model (MM). Both algorithms were computed on the web-based program, MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxent.html), which allows the calculation of strength of both acceptor and donor splice site scores. MaxEntScan requires 20 bases upstream of the 3′ splice site and the first 3 bases after the acceptor to perform 3′ splice site scoring. Three bases upstream and 6 bases downstream of the donor splice site were included to evaluate 5′ splice site scoring.[42] Exonic sequence with 25 bases of flanking intron from 10 human and mouse exons were analysed to identify putative ESEs, using ESEsfinder3.0[14, 15] for human exons, and human and mouse ESE motifs were predicted by RESCUE-ESE.[16, 17]

5.5.2 AO synthesis, design and nomenclature

2'-O-methyl modified AOs on a phosphorothioate backbone were designed to anneal to motifs predicted to be involved in pre-mRNA splicing, and synthesized in-house on and Expedite 8909 Nucleic Acid Synthesizer (Applied Biosystems Forster, CA) using the 1 μmol thioate synthesis protocol. Nomenclature was based upon that described by Mann *et al.*,[37] where the first letter designates the species (H: human, M: mouse), the number refers to the exon, the second letter indicates Acceptor or Donor Splice site and the "-" and "+" specifies intron or exon bases, respectively.

5.5.3 Myoblast culture and transfection

Normal and dystrophic human myogenic cells were prepared by a modification of the protocol described by Rando *et al.*[43] Myogenic cells were transfected with AOs at concentrations of 25 to 600 nM. Lipofectamine 2000 (Invitrogen, Melbourne, Australia) and Lipofectin (Invitrogen) were employed as transfection reagents for human myogenic cells and *H2K mdx* cells, respectively, described previously.[18]

5.5.4 RNA extraction and RT-PCR analysis

RNA was harvested from the cell cultures 24 hours after transfection using Trizol (Invitrogen), according to the manufacturer's protocol. One step RT-PCR was undertaken using 100 ng of total RNA as template in a 12.5 μl reaction for 30 cycles using 1U of Superscript III (Invitrogen). Nested PCR was carried out for 30 cycles using AmpliTaQ Gold (Applied Biosystems, Melbourne, Australia). PCR cycling conditions were performed as described by McClorey *et al* 2006.[36] PCR products were separated on 2% agarose gels in TAE buffer and the images were captured on a CHEMISMART-3000 gel documentation system (Vilber Lourmat, Marne-la-vallee, France).

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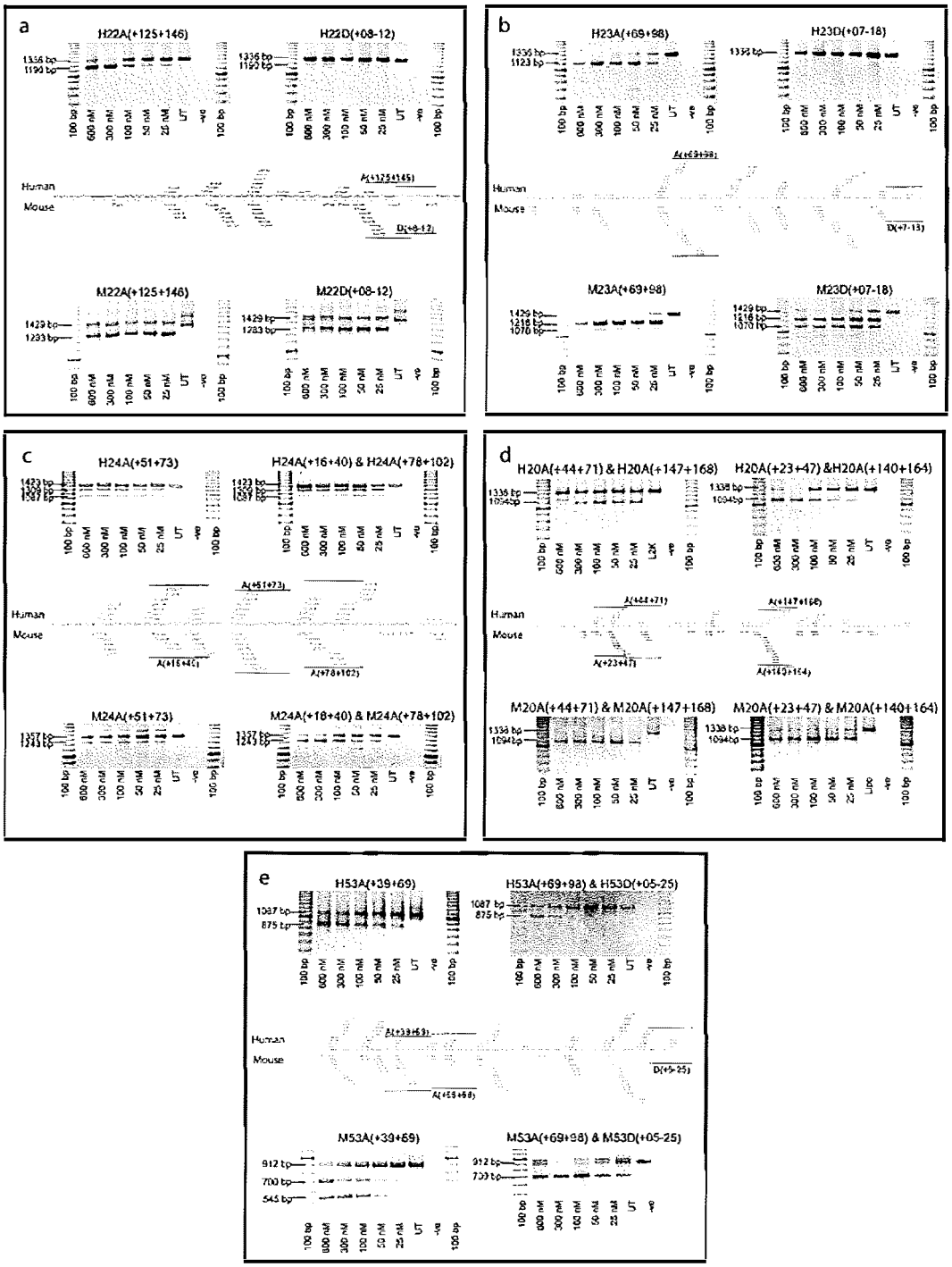

Figure 1: Patterns of dystrophin exon skipping induced by AOs targeting 5 exons ((a) 22; (b) 23; (c) 24; (d) 20; (e) 53) after transfection into normal human and *mdx* mouse myogenic cells.

Annealing coordinates relative to predicted ESEs are shown, as are the sizes of full-length and induced transcript products.

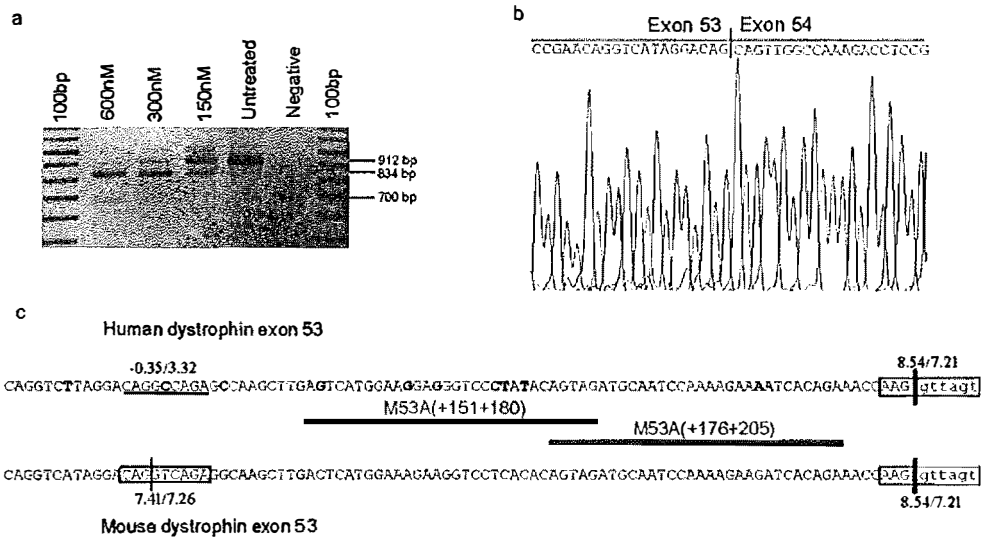

Figure 2: AO-induced cryptic splicing of mouse dystrophin exon 53.

(a) Dystrophin transcript products inAO-treated *mdx* cultures; (b) Nucleotide sequences at the novel junction arising from activation of the exon 53 cryptic donor splice site identified by DNA sequencing; (c) Partial sequence of human and mouse dystrophin exon 53 showing mouse cryptic donor splice site and AO annealing coordinates. Differences in nucleotide sequences are indicated in bold type.

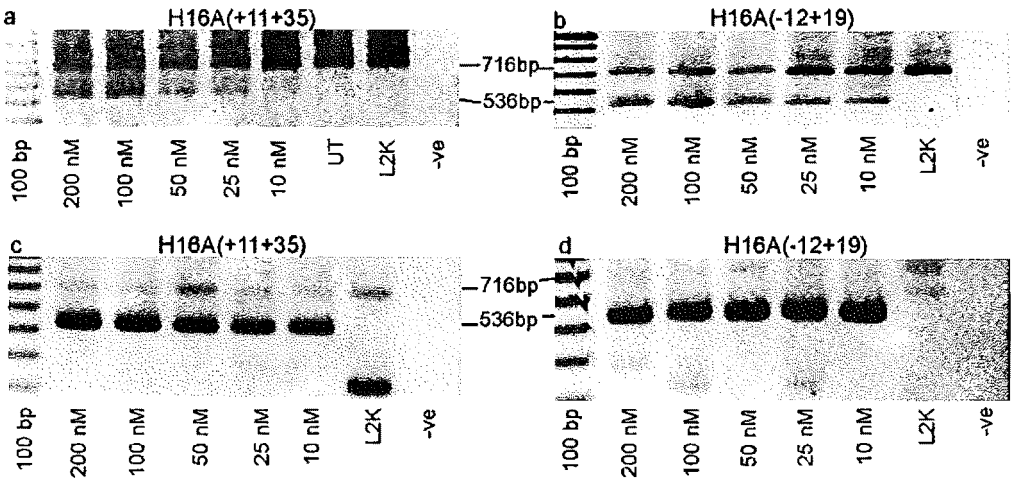

Figure 3: RT-PCR analysis of AO titration in (a and b) normal myogenic cells; and (c and d) cells from a DMD patient with a mutation (IVS16+1G>T; c.1992+1G>T) in dystrophin exon 16.

Table 1: Predicted acceptor and donor splice site scores showing length of exon and flanking introns for exons investigated.

exons	preceding intron length	exon length	following intron length	3'SS	MEM[a]	MM[a]	5'SS	MEM[a]	MM[a]
H19	16,165	88	10,236	tctttgctctcatgctgcagGCC	9.1	9.24	ATGgtaatt	6.49	5.07
M19	17035		8906	cctttgctctcatgctgcagGCC	9.16	9.07	ATGgtaatt	6.49	5.07
H20	10,236	242	6,177	aattatttttttctttctagAGG	8.65	11.04	AAGgtaaga	10.57	10.55
M20	8906		4365	tattttggttttctttgtagAGG	7.82	10.51	AAGgtaagg	10.51	10.49
H21	6,177	181	12,609	ttccatactctatggcacagGAT	4.63	6.14	CAAgtaagt	10.08	9.07
M21	4365		15930	tactctgaattatgatgcagGAT	5.28	5.8	CAAgtaagt	10.08	9.07
H22	12,609	146	3,453	ttttttccctttgataaagTTT	4.79	5.43	CAGgtctgt	6.84	6.13
M22	15930		913	tgttattctctttctttaagTTT	8.91	9.05	CAGgtctgt	6.84	6.13
H23	3,453	213	3,798	tttaaaaaattgtttttttagGCT	7.97	7.42	CAGgtaatt	8.55	7.72
M23	913		2606	aacttctatttaattttgagGCT	1.94	0.92	CAGgtaagc	9.88	11.18
H24	3,798	114	991	tataacgggtctcgtttcagAAT	8.84	6.45	AGAgtaaga	5.73	5.95
M24	2606		1096	atattgctttttttattccagAAT	7.56	10	AGAgtaaga	5.73	5.95
H25	991	156	8,606	aaattgatttatttttcttagCTT	4.36	5.32	CAGgtatag	8.73	7.25
M25	1096		5983	actatgcattgtttccatagCTT	7.16	7.46	CAGgtatga	9.46	8.96
H52	44,211	118	50,044	agggatatttgttcttacagGCA	6.66	5.58	GAAgtaagt	9.82	8.09
M52	57507		43,569	attttttttttttctttcagGCA	11.71	13.43	GAAgtaagt	9.82	8.09
H53	50,044	212	21,230	tatttttccttttattctagTTG	8.55	11.07	AAGgttagt	8.54	7.21
M53	43,569		23,325	tattcttatttttattccagTTG	7.81	10.24	AAGgttagt	8.54	7.21
H65	13,347	202	2,830	attttatttgtttttgcagTGG	8.77	11.14	TACgtacgt	6.81	6.19
M65	15253		2234	ttgtgtggtctttttgcagTGG	8.37	10.48	TACgtaagt	9.27	6.93

[a] MEM and MM are Maximum Entropy Model and First-order Markov Model, respectively.

Table 2: Nucleotide sequences of oligomers designed and evaluated for exon skipping potential. Oligomers for the human and mouse dystrophin exon are grouped by exon, light black lines separate individual AOs from AO-cocktail. ESE binding scores as predicted by ESE finder are shown the number.

Exon	Sequence (5'–>3')	Coordinates	size	SF2/ASF	SF2/ASF (IgM-BRCA1)	SC35	SRp40	SRp55
H16	CUA GAU CCG CUU UUA AAA CCU GUU AAA ACA A	H16A(-12+19)	31	3.12(2)		2.45	4.05(3)	3.07
	GAU UGC UGU UUC UUU UCU AGA UCC G	H16A(+11+35)	25	2.24		2.49(2)		3.07
HM19	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U	HM19A(+35+65)	31	2.82(2)	3.34(3)	2.40(2)	2.77	
H20	CUG GCA GAA UUC GAU CCA CCG GCU GUU C	H20A(+44+71)	28	4.62(2)	4.18(3)	4.09(4)	2.83	
	CAG CAG UAG UUG UCA UCU GCU C	H20A(147+168)	22	3.82	3.20(2)	3.63(3)	4.57(2)	
	UUG GCA GAA UUC UGU CCA CCG GCU GUU C	M20A(+44+71)						
	CGG CAG UAG UUG UCA UCU GUU C	M20A(147+168)						
M20	GUU CAG UUG UUC UGA AGC UUG UCU G	M20A(+23+47)	25	4.62(2)	4.17(2)	2.68	3.88(2)	
	AGU AGU UGU CAU CUC UUC CAA UUG U	M20A(+140+164)	25	4.22(2)	4.18(2)	3.64(3)	4.56(3)	
	GUU CAG UUG UUC UGA GGC UUG UUU G	H20A(+23+47)						
	AGU AGU UGU CAU CUG CUC CAA UUG U	H20A(+140+164)						
H21	CUG CAU CCA GCA ACA UGG GUC C	H21A(+85+106)	22	3.87(2)	3.24(2)	4.81(3)	3.37	4.35
M21	CUG CAU CCA GAA ACA UUG GCC C	M21A(+85+106)		3.87(2)	3.25	4.7(3)	4.72(2)	4.35
H22	CUG CAA UUC CCC GAG UCU CUG C	H22A(+125+146)	22	2.23	2.61(2)	4.21(2)	3.90(2)	2.95
	CUG UAA UUU CCC GAG UCU CUC C	M22A(+125+146)						
M22	AUG UCC ACA GAC CUG UAA UU	M22D(+08-12)	20	2.68(2)	2.72	4.21	6.01(2)	3.41
	AUA UUC ACA GAC CUG CAA UU	H22D(+08-12)						
H23	CGG CUA AUU UCA GAG GGC GCU UUC UUC GAC	H23A(+69+98)	30	2.04	2.32(3)	3.66		
	UGG CAU AUU UCU GAA GGU GCU UUC UUG GCC	M23A(+69+98)						
M23	GGC CAA ACC UCG CCU UAC CUC AAA U	M23D(+07-18)	25	3.65(2)	3.9		4.97	
	AGU AAA AUC UUG AAU UAC CUG AAU U	H23D(+07-18)						
H24	CAA GGG CAG GCC AUU CCU CCU UC	H24A(+51+73)	23	2.79(2)	2.31(3)	2.67	2.98	
	CCA GGG CAG GCC AUU CCU CUU UC	M24A(+51+73)						
M24	CAA CUU CAG CCA UCC AUU UCU GUA A	M24A(+16+40)	25	2.34	2.45	3.02	3.63	3.74
	GAG CUG UUU UUU CAG GAU UUC AGC A	M24A(+78+102)	25			4.31(2)		
	CAA CUU CAG CCA UCC AUU UCU UCA G	H24A(+16+40)						
	CAG CUG CUU UUU UAG AAU UUC UGA A	H24A(+78+102)						
H25	UUC AGU UCU GUC UCA AGU CUC CAA G	H25A(+95+119)	25	2.29		3.16(2)		3.83
	CUA AGU UCU GUC UCC AGU CUG GAU G	M25A(+95+119)		3.50(2)	2.94	3.02	4.21	5.53

Table 2: Nucleotide sequences of oligomers designed and evaluated for exon skipping potential. Oligomers for the human and mouse dystrophin exon are grouped by exon, light black lines separate individual AOs from AO-cocktail. ESE binding scores as predicted by ESE finder are shown the number (Continued).

H52	UCC AAC UGG GGA CGC CUC UGU UCC AAA UCC	H52A(+12+41)	30	3.53(2)	2.74(4)	4.78(3)	3.55	3.33(2)
	UCC AAU UGC GGG CCU CUC UGU UCC AAA UCU	M52A(+12+41)						
M52	UCC AAU UGG GGG CGU CUC UGU UCC A	M52A(+17+41)	25	2.56	2.54(2)	3.33(3)		
	UUC AAA UUC UCC GCA GCA GUA AUG AGU UCU	M52A(+42+71)	30		2.73(2)	2.91(2)	4.92	3.02
	UCC AAC UGG GGA CGC CUC UGU UCC A	H52A(+17+41)						
	UUC AAA UUU UCG GCA GCG GUA AUG AGU UCU	H52A(+42+71)						
H53	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G	H53A(+39+69)	31	3.08(2)	3.69		3.19	
	CAU UCA ACU GUU GUC UCC UGU UCU GCA GCU G	M53A(+39+69)						
M53	CAG CCA UUG UGU UGA AUC CUU UAA CAU UUC	HM53A(+69+98)	30			2.94(2)	3.46(2)	
	UUU UAA AGA UAU CCU UGA CAC UAA CCU UGG	M53D(+05-25)	30		2.52	4.26	3.89	3.28(2)
	UUA AAA AGC UAU CUU UGA UAC UAA CCU UGG	H53D(+05-25)						
	CUA CUG UGU GAG GAC CUU CUU UCC AUG AGU	M53A(+151+180)	30	3.86(3)	3.65(3)	4.58(2)	3.64(3)	
	UCU CUC AUC UUC UUU UGC AUU GCA UCU ACU	M53A(+176+205)	30	3.41	3.41	4.03	3.94(2)	2.91
H65	GCU CAA GAG AUC CAC UGC AAA AAA C	H65A(-11+14)	25	3.29	3.15	2.52(2)	3.01	
	GUU GUG CUG GUC CAA GGC AUC ACA U	H65A(+26+50)	25			3.61	4.35	3.76
	GCU CAA GAG AUC CAC UGC AAA AAA G	M65A(-11+14)						
	GUU GUG CUG GUC CAG GGC AUC ACA U	M65A(+26+50)						
HM65	UCU GCA GGA UAU CCA UGG GCU GGU C	HM65A(+63+87)	25	2.77	2.97(3)	3.42(2)	3.20	4.35

CHAPTER 6

Antisense oligomer-induced exon inclusion to treat spinal muscular atrophy

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6.1 Abstract

Spinal muscular atrophy (SMA) is due to a deficiency in the survival of motor neuron (SMN) protein, most commonly caused by loss of the entire SMN1 gene. Absence or reduced amounts of the SMN protein result in degeneration of the anterior horn cells and clinically to hypotonia, symmetrical muscle weakness and atrophy, tremor of finger and hand, and fasciculation of the tongue muscles. Although the SMN1 gene homologue, SMN2, is present in SMA patients, a single base (C/T) difference at the sixth nucleotide in exon 7 of the SMN2 gene promotes excision of that exon from the mature transcript, thereby dramatically reducing the amount of full-length SMN transcript and SMN protein. The full-length SMN2 product partially compensates for the lack of SMN1 protein in SMA.

Given the molecular basis of SMA, this paper explores whether antisense oligomers (AOs) provide a way of enhancing the number of SMN transcripts containing exon 7 in cultured fibroblasts from an SMA patient. By masking silencing splice motifs and promoting exon 7 inclusion, the SMN 2 transcript could be translated into a functional protein.

Our results suggest that masking the potential intronic silencing motifs, located in SMN2 introns 6 and 7, by the use of AOs, promotes exon inclusion as determined by RT-PCR. Combining selected oligomers into multi-AO cocktails enhanced exon 7 inclusion in a cumulative and dose dependent manner. Similarly, changing the chemistry of AOs from 2'-O-methyl oligomers on phosphorothioate backbone to phosphorodiamidate morpholino oligomers, further enhanced AO-induced exon inclusion and led to SMN protein expression. This study provides additional insights into possible intronic splice silencing motifs, which could be targeted in combination with previously identified motifs, as candidates for a molecular therapeutic approach to SMA, and may perhaps open new avenues for the treatment of other genetic disorders where oligomers could be used to induce exon inclusion.

6.2 Introduction

Spinal muscular atrophy (SMA) is the most common autosomal recessive neurodegenerative disorder in infants and children, with an incidence of 1 in 10,000 live births, and a carrier frequency of 1 in 40-50 in adults.[1, 2] The disorder is characterised by degeneration of the anterior horn cells leading to four main symptoms, hypotonia, symmetrical muscle weakness and atrophy, tremor of finger and hand, and fasciculation of the tongue muscles.[3]

In SMA patients, the morphology and function of motor neurons show signs of immaturity, with a reduced number of motor neurons in the ventral horn cells the most striking pathological finding (**Fig 1**). Most surviving motor neurons exhibit some signs of immaturity, including smaller size and less-developed granular endoplasmic reticulum (Nissl body) compared to normal individuals of comparable age. In the motor axons, the ventral nerve roots of SMA patients contain a relatively high proportion of poorly myelinated thin axons and multi-axonal bundles of low density (based on axon per mm^2). Axons in peripheral nerves show extended latency to electrical stimulation and reduced conduction velocities (for review see[4, 5]).

Spinal muscular atrophy arises from a deficit in the survival of motor neuron (SMN) protein, which in most patients is caused by mutations in the SMN1 gene.[6, 7] Deletions, point mutations, and gene conversion in both alleles of the survivor motor neuron 1 gene (SMN1) are responsible for more than 94% cases of SMA with drastically reduced levels of SMN protein.[8, 9] There are two copies of the SMN gene, SMN1 (telomeric SMN) and SMN2 (centromeric SMN)[6] (**Fig 2**), but little SMN protein is produced by SMN2. A single base (C/T) difference at the sixth nucleotide in exon 7 of the human SMN2 gene is known to cause exon 7 skipping, thereby drastically reducing the amount of SMN protein.[10] The T base of SMN2 has been proposed either to cause disruption of an exonic splice enhancer (ESE) or create a novel splice silencing motif (**Fig 3**). The full-length product transcribed from SMN2 partly compensates for lack of the SMN1 gene product in milder forms of SMA (SMA types II and III).

While there is still some debate about how reduced amount of SMN protein leads to the pathophysiology of SMA[11], several mechanisms have been proposed. One mechanism is believed to involve a deficit in snRNP biosynthesis of the SMN protein. SMN protein is an important component of the SMN complex,[12] where assembly of the snRNAs and common core proteins of the snRNP complex (Sm protein) occurs. This complex also has a role in ensuring the authenticity of snRNA binding to the Sm protein

allowing the formation of the snRNP complex to proceed.[13] A lack of the SMN protein causes tissue-specific perturbation in the expression of snRNAs and gene mis-splicing.

A second mechanism has been postulated from the observation that SMA is associated with the predominant expression of a truncated SMN transcript containing no exon 7. The absence of exon 7 from the SMN transcript encoding a domain critical to normal SMN function not only results in the degradation of the truncated SMN protein, but may also confer pro-apoptotic activity.[14]

A third proposed mechanism put forward to explain disease pathophysiology is that a lack of SMN protein leads to drastic loss of motor neurons and a defect of axonogenesis. The lethality of most SMN knockout and knockdown animal models suggests that the SMN protein complex is critical to normal development.[15-17] Recently, under non-lethal knockdown conditions, zebrafish embryos were shown to undergo development but exhibited dramatic SMA-like motor axon degeneration.[18] Furthermore, the injection of purified spliceosomal U snRNPs into SMN-deficient embryos forestalled developmental arrest in *Xenopus* and prevented motor neuron degeneration in zebrafish.[18] These findings add weight to the critical importance of defective axonogenesis in disease pathophysiology.

Depending upon the onset and severity of disease, patients with SMA are classified as SMA types I, II or III. SMA type I is the most severe form, resulting in death in infancy, whilst the milder forms, SMA types II and III are associated with extra copies of the SMN2 gene.[19, 20] The disease phenotype is now recognised to be dependent on the levels of full-length SMN protein. An approach aimed at enhancing SMN2 expression has been pursued as a genetic treatment for SMA patients, albeit with limited success.[21-25]

The use of antisense oligomers (AO) as a genetic intervention for SMA has been pursued as another way of enhancing the number of SMN2 transcripts containing exon 7. Theoretically, by masking silencing splice motifs or strengthening exon 7 recognition, the SMN 2 transcript should be translated into a functional protein if exon 7 forms part of the mature transcript. Several studies have sought to identify those silencing splice motifs, where binding of AOs can promote exon 7 inclusion and SMN protein, and ultimately be clinically beneficial to SMA patients.[1, 26-29] Here, we examine the effect of masking potential intronic silencing motifs by the use of AOs in cultured fibroblasts from an SMA patient. As combinations of oligomers were shown to synergistically induce exon skipping in human and mouse dystrophin gene

transcripts,[30-32] AO-cocktails were investigated with the goal of maximising the efficiency of exon 7 inclusion. Studies on the effects of exon inclusion after using AOs with different backbone chemistries are also reported.

6.3 Results

6.3.1 Preliminary evaluation of AO-induced exon inclusion

Two exonic and one intronic silencing motifs on SMN2 pre-mRNA have been previously identified as potential target regions capable of enhancing production of the full-length SMN2 transcript.[1, 29] AOs α[28], f[1] and g[1] targeting these motifs were evaluated for their ability to induce exon 7 inclusion in cultured fibroblasts from an SMA patient. Only AO-α (SMN2D-10-29) was shown to substantially induce exon 7 inclusion at the RNA level, as determined by RT-PCR analysis (**Fig 4a, b**). An array of 22-mer-AOs (AOs b, c, d and e), designed to anneal to differential positions in the vicinity of -10 to -29 on the exon 7 donor splice site on pre-mRNA of SMN2, was evaluated to establish whether the efficiency of exon 7 inclusion could be improved by targeting different sites. However, use of these AOs led to no detectable improvement in exon 7 inclusion compared to AO-α.

6.3.2 Optimising AO- induced exon inclusion

Sixteen oligomers were designed to target motifs within either intron 6 or 7 adjacent to either acceptor (A1-A8) or donor (D1-D8) splice sites, with the goal of masking any potential intronic splice silencers and inducing expression of SMN2 transcript containing exon 7. The effects of these AOs were compared with that of AO-α, which were previously reported by Singh *et al.*,[29] in cultured SMA fibroblasts. Whereas AO-α treatment caused >90% exon inclusion, transfection of cultured fibroblasts with AOs A3, A5, A6, D3, D4, D6 or D7 promoted exon 7 inclusion, albeit to less than 80%. Only in the case of treatment with AOs A3, A5 or A6 was the effect dose dependent (**Fig 5**). Other oligomers led to either minimally induced exon inclusion or exon skipping (**Fig 5**).

Three regions to which binding of AOs (A3, A5, A6, D3, D5 or D6) resulted in substantial exon 7 inclusion were selected for further optimisation. These were β1 (-90 to -51 upstream from acceptor site of exon 7), β2 (-75 to -94 downstream from donor splice site of exon 7) and β3 (-135 to -174 downstream from donor splice site of exon 7) (**Fig 6a**).

Three 25mers (AOs 10, 11 and 12), designed to anneal to the β1 region on pre-mRNA of SMN2 were found to be less active in inducing exon 7 inclusion compared to A6 treatment (**Fig 6**). Evaluating AOs of variable length led to differences in inclusion of exon 7, with two 25mers (A9 and A10) being less effective than A6 (20mer) (**Fig 6**). In the β2 region, five nucleotide extension of D3 at either the 5' or 3' end induced exon 7 inclusion as efficiently as in D3 transfected cultures (**Fig 6**), however, for the β3 region, additional bases at either end of D6 led to reduced exon 7 inclusion compared to D6- treated SMA-fibroblasts (**Fig 6**). The D7 oligomer was found to be more efficient compared to the derived 30mer (D13) and 25mer (D14).

6.3.3 Optimising AO-cocktail induced exon 7 inclusion

Two oligomers targeting intron 6 and three oligomers annealing to intron 7 were selected to evaluate whether a combination of AOs at different concentrations (150, 300 and 600 nM) can further enhance exon 7 inclusion (**Fig 7**). A number of two-AO cocktails were shown to lead to substantial exon 7 inclusion in a concentration-dependent manner. The two-AO cocktails (A6+D3, A6+D6, A6+D7, D3+D6, D3+D7, D6+D7 and A3+D3) were found to be marginally superior to the combinations (A3+D6 and A3+D7) (**Fig 7**). The combination of A6+D6 led to cumulative exon inclusion compared to individual treatment of fibroblasts with A6 and D6 (**Fig 7**). Treatment of fibroblasts with three-AO cocktails (A3+D3+D6, A3+D3+D7 and A3+D6+D7) resulted in comparable levels of exon inclusion compared to the A6+D6 cocktail.

6.3.4 Effects of different backbone chemistries on AO induced exon 7 inclusion

PMO-α, conjugated to cell penetrating peptide (P007), was synthesised with the same nucleotide sequence as AO-α, and its efficiency in inducing exon 7 inclusion compared with that of AO-α, complexed with cationic liposome, in cultured SMA fibroblasts. Based on RT-PCR and densitometric of the products, 200 nM of AO-α induced exon 7 inclusion in approximately 70% of the SMN transcripts by day 5 (**Fig 8a and b**). However, at day 7 post AO-α transfection, the level of induction of SMN transcripts containing exon 7 was no greater than that of untreated SMA fibroblasts (**Fig 8a and b**). Treatment with PMO-α at 1 μM, by contrast, led to 100% exon inclusion at day 7 after treatment (**Fig 8a**). SMN transcripts were not detectable at day 10 after AO-α transfection due to poor cell viability (**Fig 8a**). PMO-α treatment led to a 2-fold increase in the level of SMN protein by day 7, as determined by densitrometric analysis of western blots (**Fig 8c and d**). In contrast, no increase in SMN protein compared to

untreated fibroblasts was observed at day 5 after AO-α treatment (**Fig 8c**).

6.4 Discussion

Several studies aimed at identifying exonic and intronic splice motifs on the pre-mRNA of the SMN gene have been reported, where the goals were (i) understanding of the complex regulatory interplay between *cis*-acting elements and *trans*-factors, and (ii) developing a molecular treatment for SMA patients.[1, 28, 29, 33] The C/T nucleotide transition has been the main focus for studies seeking to understand why SMN2 pre-mRNA does not predominantly produce full-length transcripts containing exon 7. Two different splice motifs, an ESE on SMN1[34] and an ESS on SMN2[35] have been identified and proposed as reasons for differences in the levels of full-length transcripts between SMN1 and SMN2 products. Intronic silencing motifs have also been identified using mutagenesis of an artificial SMN construct.[29]

In the present study, two exonic and one intronic silencing motifs, which have been previously reported as possible targets for enhancing production of the full-length SMN2 transcript[1, 29], were used as the initial targeted regions for investigation of exon inclusion. Targeting the two exonic silencers led to no change in exon 7 inclusion in cultured fibroblasts from an SMA patient. Based upon RT-PCR analysis, 37.5-150 nM AO-α (SMN2D-10-29) was shown to induce substantial exon 7 inclusion at the RNA level. The use of an array of oligomers (AOs b, c, d and e) annealing to adjacent positions along intron 7, starting from 10 nucleotides downstream of the exon 7 donor splice site on SMN2 pre-mRNA led to no improvement in exon inclusion, compared to AO-α.

Human and mouse regulatory splice motifs are conserved to some degree,[36-38] and are found close to the splice junction and up to 200 nucleotides away.[39] We explored the potential intronic splice silencing motifs in regions within 200 nucleotides either upstream or downstream from the acceptor or donor motifs of exon 7 on SMN2 pre-mRNA. Sixteen oligomers were designed to target either intron 6 or 7 at locations adjacent to either acceptor (A1-A8) or donor (D1-D8) motifs. Whereas AO-α treatment caused >90% exon inclusion at 150 nM, transfection of cultured fibroblasts with several AOs (A3, A5, A6, D3, D6 or D7) at 150 nM caused exon inclusion between 70 to 80%. Other oligomers led to either minimally induced exon inclusion or even promoted exon exclusion. The efficiency of AOs targeting the newly identified intronic splice silencing motifs was no greater than that of AO-α in inducing exon 7 inclusion.

Three targeted regions β1 (-90 to -51 upstream from the acceptor site of exon 7), β2 (-75 to -94 downstream from donor splice site of exon 7) and β3 (-135 to -174 downstream from donor splice site of exon 7) were selected on the grounds of their efficiency in promoting exon 7 inclusion. Modifying AOs by size extension, either at the 5', 3' or both ends of oligomers targeting the three selected regions (β1, β2 and β3), led to no marked change in the efficiency of AO-induced exon 7 inclusion.

Although, binding of hnRNP A1 to either exonic or intronic cis-elements was reported to cause exon 7 skipping in the SMN 2 gene transcript,[35] of five AOs (AOs f, A6, A7, D4 or D7) masking the hnRNP A1 splice silencing motif (UAGNNA/U), only two AOs (A6 or D7) increased full-length SMN transcripts. A retrospective study using the putative exonic splicing enhancers/silencers program[40] revealed thirteen splice silencing motifs, located in introns 6 and 7 and exon 7 of human SMN 2 pre-mRNA, however these did not correlate with AO efficiency. As shown in **Table 1**, there is also no obvious difference in the percentage of GC content and melting temperature (Tm) of effective AOs compared to those that were inefficient.

Two oligomers targeting intron 6 and three oligomers annealing to intron 7 were selected for evaluation in combinations, to determine whether AO-cocktails could further enhance exon 7 inclusion. Combining A6 and D6 enhanced exon inclusion 7, compared to individual treatment of fibroblasts with either A6 or D6. Most two-AO cocktails (A6+D6, A3+D3, A6+D3, A6+D7, D3+D6, D3+D7 and A6+D7) caused substantial exon 7 inclusion in a concentration-dependent manner.

It has been proposed that two hnRNP A1s promote 'looping out' in pre-mRNA structure and cooperatively induce exon exclusion from the mature mRNA.[41] A cocktail of AOs A6+D7, masking two hnRNP A1 binding motifs flanking exon 7, resulted in equivalent levels of exon 7 inclusion in SMN 2 gene transcript compared to most two-AO cocktails.

Treatment of SMA fibroblasts with three-AO cocktails (A3+D3+D6, A3+D3+D7 and A3+D6+D7) resulted in comparable levels of exon inclusion, compared to the A6+D6 cocktail. However, both two- and three-AO cocktails resulted in approximately 85% exon inclusion at a combined concentration of 150 nM, whereas treatment with AO-α led to 95% exon inclusion at an equivalent concentration.

In addition to optimising the composition of AO-cocktails, we also investigated whether changing the oligomer backbone chemistry affects the level of AO-induced

protein expression, by comparing the effect of 2OMeAO (AO-α) with that of PMO-α. AO-α induced exon inclusion peaked at day 2 (90%) and thereafter declined to 70% at day 5 and to levels no greater than those of untreated SMA fibroblasts by day 7. No SMN transcripts were detectable at day 10 after AO- α transfection. This pattern is similar to previous reports from this laboratory on the effects of 2OMeAO in inducing exon skipping, where levels were highest at 24-48 hours after transfection, but were greatly reduced over the next 9 days.[42] This is most likely due to nuclease degradation of the 2OMeAO.[43]

Treatment with PMO-α at 1 μM, by contrast, resulted in 100% of full-length SMN transcripts containing exon 7, and increased levels of SMN protein at day 7 after treatment, while AO-α treatment of cultured fibroblasts led to no increase in the level of SMN protein compared to untreated fibroblasts. Likewise, there was a high degree of variability in the inductions of dystrophin protein in canine cultured cells from the Golden Retriever model of muscular dystrophy and in human dystrophic muscle explants,[44, 45] whereas use of PMOs with the same nucleotide sequences led to highly consistent dystrophin protein expression at 7 days after transfection.[45] PMOs appear to be the compounds of choice for clinical trials to treat DMD patients because of their long half-life and relatively apparent low toxic.[46, 47]

In summary, selected oligomers were shown in this study to be capable of increasing SMN2 exon 7 inclusion, at least in cultured fibroblasts from an SMA patient. Moreover, combining AOs into cocktails enhanced levels of the full-length SMN transcript, containing exon 7 in a cumulative and dose-dependent manner. However AO-cocktails induced exon 7 inclusion to a lesser degree than AO-α. Finally, changing the backbone chemistry of oligomers by using PMOs further enhanced exon 7 inclusion and led to substantial SMN protein expression. Unlike 2OMeAOs, PMOs are not subjected to nuclease degradation, and do not require a lipid carrier, which may cause cellular toxicity. Moreover, conjugation of a PMO with cell penetrating peptide tags facilitates oligomer delivery. Summation of these advantages contributes to enhancement of exon 7 inclusion in PMO-treated SMA cells. In conclusion, this study provides additional information about possible intronic splice silencing motifs, which could be used in combination with previously identified motifs, as sites for a molecular therapeutic approach to SMA, and may perhaps open up new avenues for the treatment for other genetic disorders where oligomers could be used to induce exon inclusion.

6.5 Materials and methods

6.5.1 AOs and primers

The sequences of the AOs used and their relative coordinates are shown in **Table 1**. 2OMeAOs were synthesised on an Expedite 8909 Nucleic Acid synthesizer using the 1 μmole thioate synthesis protocol. AOs were designed to anneal to either putative intronic or exonic splice silencer motifs of human SMN2 pre-mRNA exon 7. PMOs conjugated to an arginine-rich, cell penetrating peptide (P007)[48, 49] were synthesized by AVI BioPharma Inc. (Corvallis, OR, USA). Primers for RT-PCR and sequencing analysis were synthesised by Geneworks (Adelaide, Australia) and are listed in supplemental information.

6.5.2 Cell culture and AO transfection

Cultured fibroblasts from an SMA type I patient were seeded and proliferated into 75 cm^2 tissue culture flasks. When nearly confluent, cells were seeded into 24 well-plates and incubated for 24 hours before transfection. Duplicate wells were transfected with 2OMeAO lipoplexes using Lipofectamine (Invitrogen) at an equivalent amount of Lipofectamine to AO. Briefly, Lipofectamine was mixed with DMEM (Invitrogen) to a final volume of 200 μl and incubated for 10 minutes at room temperature. The 2OMeAO, which had been prepared in 200 μl DMEM, was then combined with Lipofectamine:DMEM and the mixture incubated for a further 20 minutes, before addition of DMEM to a final volume of 1 ml and subsequent addition of 500 μl aliquots to each well. Transfected cells were incubated for 48 hours before RNA was extracted for analysis.

6.5.3 RNA extraction, RT-PCR analysis, and DNA sequencing

RNA was harvested from cultured SMA fibroblasts using Trizol (Invitrogen), according to the manufacturer's protocol. One-step RT-PCR was undertaken using 120 ng of total RNA as template, in a 12.5 μl reaction for 28 cycles of amplification. After the reverse transcription step for 30 minutes at 55°C, the reaction was heated to 94°C for 2 minutes before the primary thermal cycling rounds of 94°C for 40 seconds, 56°C for 1 minute, and 68°C for 1 minute. PCR products were separated on 2% agarose gels in TAE buffer pH 8.3, and the images captured on a CHEMISMART-3000 (Vilber Lourmat, Marne-la-vallee, France) gel documentation system. Bands of interest were re-amplified directly from the agarose gel[50] and the sequencing templates were purified using UltraClean spin columns (Mobio Laboratories, CA, USA) and then sequenced on

a 3730 ABI DNA sequencer using BigDye v3.1 terminator chemistry (Applied Biosystems).

6.5.4 Western blot analysis

Protein extracts were prepared by adding an equal volume (w/v) of treatment buffer containing 125 mM Tris-HCl, pH 6.8, 15% sodium dodecyl sulphate, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM dithiothreitol, bromphenol blue, and protease inhibitor cocktail (Sigma) to a cell pellet. Samples were then vortexed and sonicated briefly, heated at 95°C for 5 minutes, before electophoretic fractionation on a 4 to 10% SDS gradient gel at pH 8.8 with a 4% stacking gel, pH 6.8. Densitometry of protein bands after coomassie blue staining was undertaken to enable calculation of protein levels. Extracts from AO-treated cultures were loaded onto a second PAGE gel for western blotting. Proteins were transferred from the gel to nitrocellulose membranes (Amersham Biosciences, Castle Hill, Australia) for 4 hours at 18°C at 290 mA. SMN was visualised using anti-SMN polyclonal antibody (Santacruz, UK)[51]. Images were captured on a Vilber Lourmat CHEMISMART-3000 gel documentation system. Levels (%) of SMN were calculated relative to those in untreated cells after normalisation for total protein loading.

6.6 References

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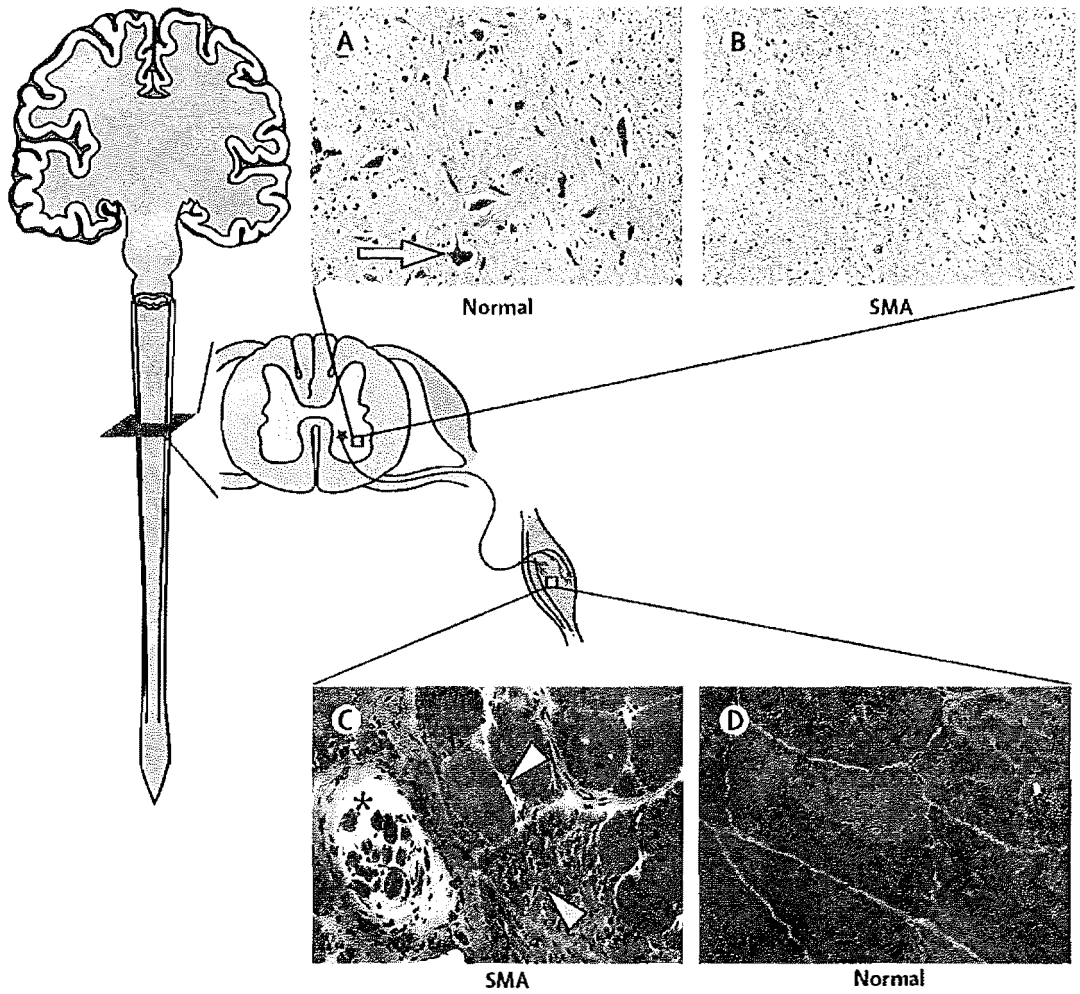

Figure 1: Histopathology of spinal muscular atrophy.

Normally, neuronal signals are transmitted from the motor cortex to muscle fibres through α-motor neurons. The green arrow in (A) shows an α–motor neuron in a spinal cord section from a normal human. Motor stimuli are not transmittable to muscle fibres in SMA patients because of a lack of α-motor neurons as shown in a spinal cord section from an SMA patient (B). Hypertrophic fibres (shown by a hollow arrowhead) surrounded by group atrophy (shown by a green arrowhead) are histopathological features of type I SMA-derived muscle sections stained with haematoxilin and eosin (see C) compared to the morphologically uniform muscle sections seen in normal humans (D). (modified from Lunn *et al.* 2008[5])

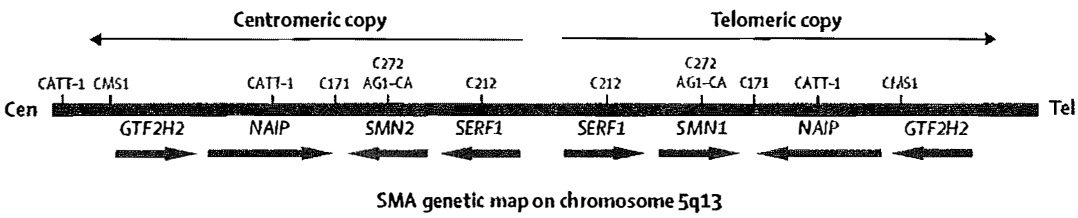

Figure 2: Genetic location of the survivor motor neuron (SMN) gene.

A defect which is responsible for spinal muscular atrophy (SMA). The figure shows that two large inverted genomic fragments (black horizontal lines) on chromosome 5q13 are the sites of the two SMN genes. The telomeric copy (SMN1) is transcribed toward the telomere as denoted by the red arrows, whereas at least one centromeric copy of SMN2 is transcribed toward the centromere as indicated by the blue arrows. Other genes in the proximity of the SMN gene copies are shown in black and multiple copies of the microsatellite markers (red) are indicated within these regions. (from Lunn *et al.* 2008[5])

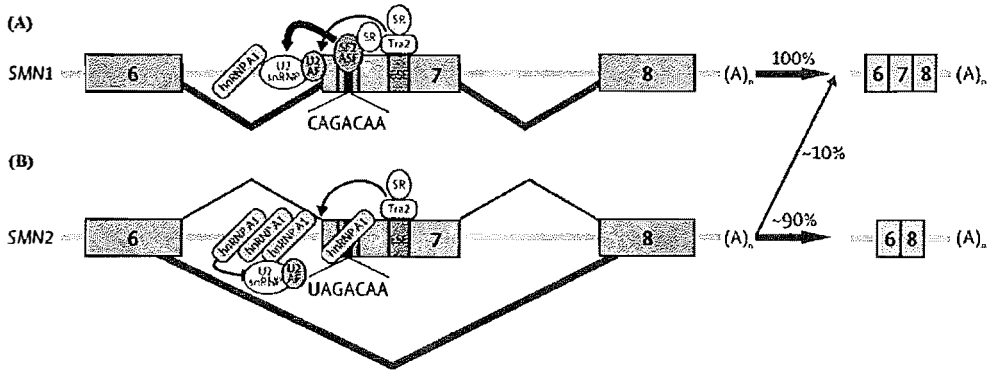

Figure 3: Regulation of exon 7 pre-mRNA splicing of the SMN1 and SMN2 genes.

(A) In SMN1 splicing, stimulation of exon 7 inclusion is controlled by two mechanisms, one mediated by Tra2 through its interaction with U2AF trans-factor and the second by splicing factor 2 (SF2) and the complex it forms with alternative splicing factor (SF2/ASF), which stabilises binding of the U2 small nuclear ribonuclear protein (U2 snRNP) to pre-mRNA. Binding of two trans-factors to exonic splice enhancers (ESEs) leads to exclusive exon 7 inclusion into the SMN mature transcript. (B) In SMN2 splicing, approximately 10% of the full-length transcript containing exon 7 is transcribed from the SMN2 gene because of the substitution of a cytosine with a thymidine at the sixth nucleotide from the acceptor of exon 7 on pre-mRNA. Two proposed models may explain why the major spliced product of the SMN2 excludes exon 7. One proposal is that this is caused by disrupting SF2/ASF binding, whereas the second model suggests that it is mediated by binding of heterogeneous nuclear ribonuclear protein (hnRNP) A1, which results in about 90% of the SMN2 spliced transcripts containing no exon 7. The Tra2 protein complex on exon 7 accounts for approximately 10% of full-length SMN2 transcripts (modified from Lunn *et al.* 2008[5]).

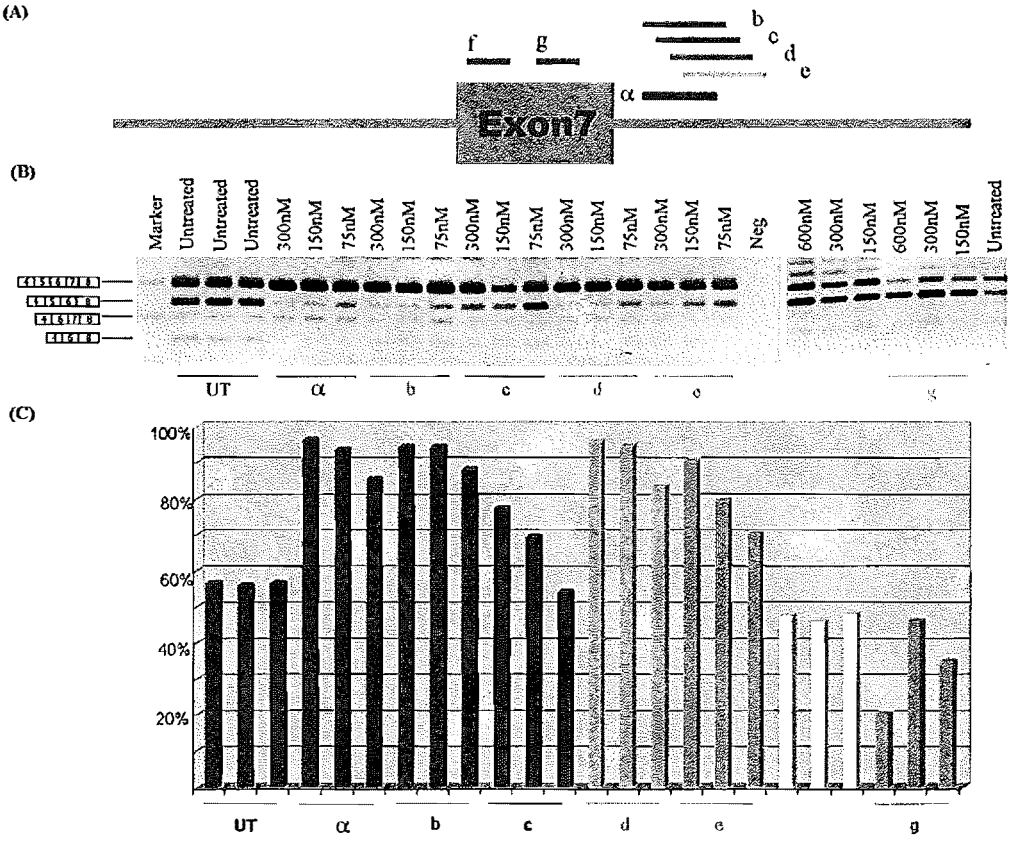

Figure 4: Relative annealing coordinates of oligomers to pre-mRNA of SMN2 and their efficacy to induce exon 7 inclusion in cultured fibroblasts from an SMA patient.

(A) Relative annealing coordinates of selected oligomers to exon 7 and the adjacent intron 7. Oligomers α, f and g were previously identified as candidates for AO-induced exon inclusion. Four additional "micro-walk" oligomers were designed to establish the optimal annealing coordinates for exon 7 inclusion. (B) RT-PCR analysis of cultured fibroblasts from a SMA patient treated with the different oligomers (α, b-g) at different concentrations (75, 150 and 300nM). Also shown are results from untreated patient fibroblasts and a negative (saline) control. The size of the four isoforms (viz. exons 4+5+6+7+8, exons 4+5+6+8, exons 4+5+7+8 and exons 4+5+8) of SMN2 and the two isoforms (viz. exons 4+5+6+7+8 and exons 4+5+6+8) of SMN1 were determined using molecular weight markers. (C) Levels of the full-length SMN1 and SMN2 transcripts containing exon 7 as determined by densitrometric analysis of RT-PCR, relative to the levels of the two major isoforms (exons 4+5+6+7+8 and exons 4+5+6+8) after treatment with the different oligomers (α, b-g) at different concentrations (75, 150 and 300 nM).

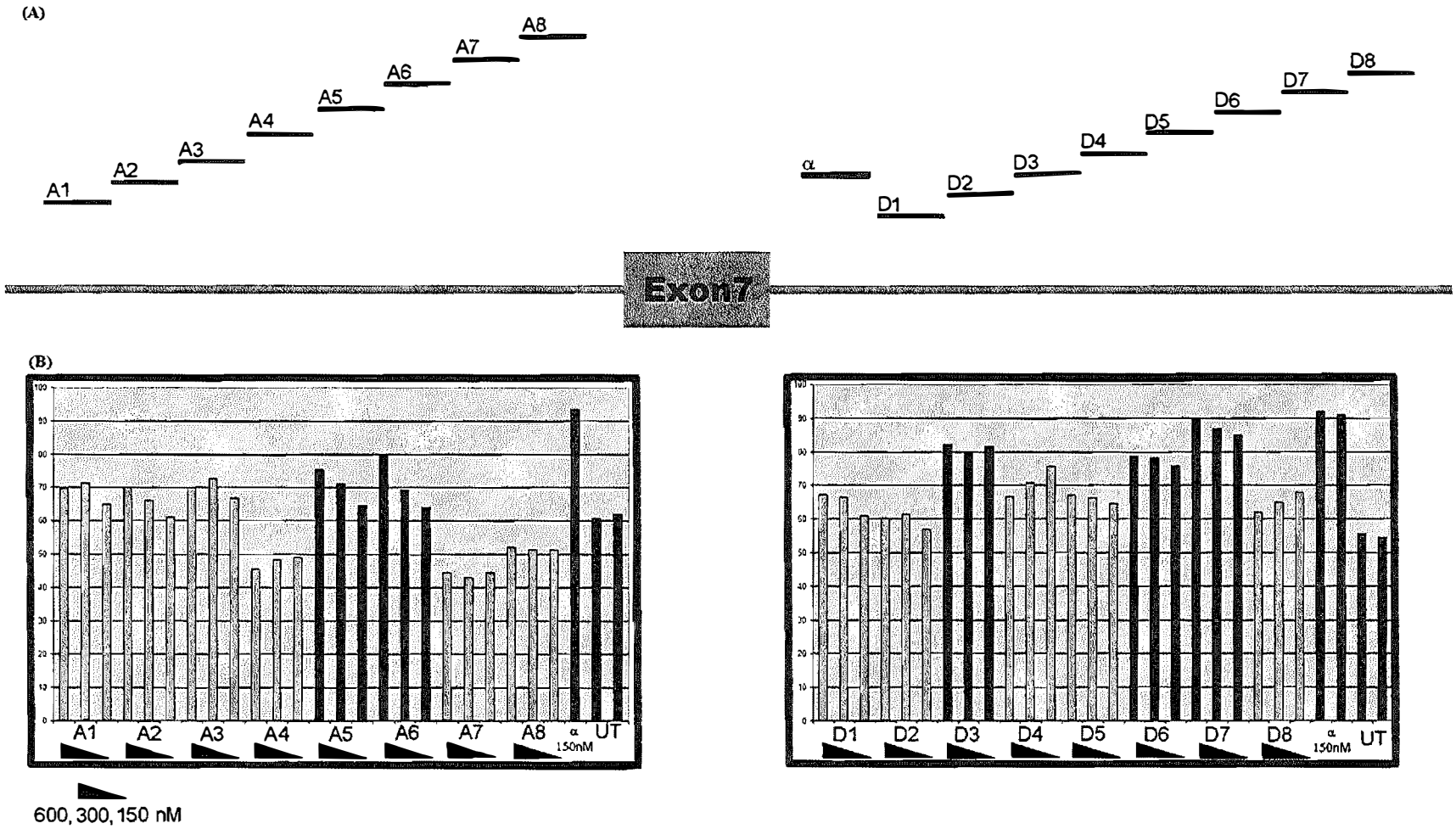

Figure 5: Relative annealing coordinates of oligomers targeting intronic regions of SMN2 pre-mRNA and their efficacy in inducing exon inclusion.

(A) Relative annealing coordinates of seventeen selected oligomers on the region flanking exon 7 in the SMN2 pre-mRNA. (B) Levels of full-length SMN1 and SMN2 transcripts containing exon 7, as determined by densitrometric analysis of RT-PCR of cultured fibroblasts from an SMA patient treated with oligomers (A1-A8, D1-D8 or α) targeting introns 6 and 7 at various concentrations (150, 300 and 600nM), relative to the levels of the two major isoforms (exons 4+5+6+7+8 and exons 4+5+6+8).

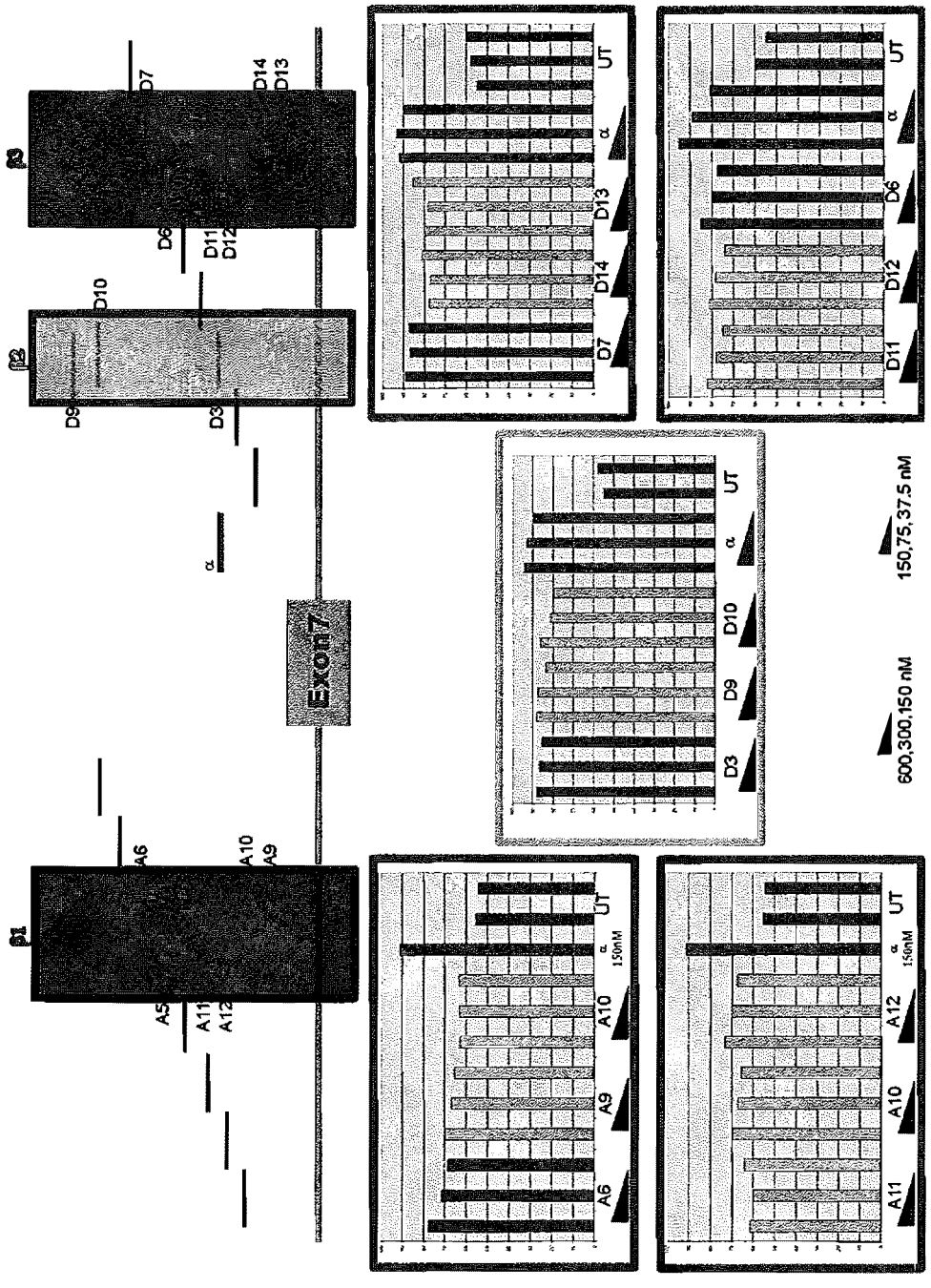

Figure 6: Exon inclusion efficiency of oligomers selected to induce exon 7 inclusion.

(A) Relative annealing coordinates of selected oligomers targeting β1 (-90 to -51 upstream from the acceptor site of exon 7), β2 (-75 to -94 downstream from the donor splice site of exon 7); and β3 (-135 to -174 downstream from the donor splice site of exon 7). (B) Levels of full-length SMN1 and SMN2 transcripts containing exon 7, as determined by densitrometric analysis of RT-PCR on RNA from cultured fibroblasts from an SMA patient treated with oligomers (A5, A6, A9-A12, D6, D7, D9-D14 and α) targeting selected regions at different concentrations (150, 300 and 600nM), relative to levels of the two major isoforms (exons 4+5+6+7+8 and exons 4+5+6+8).

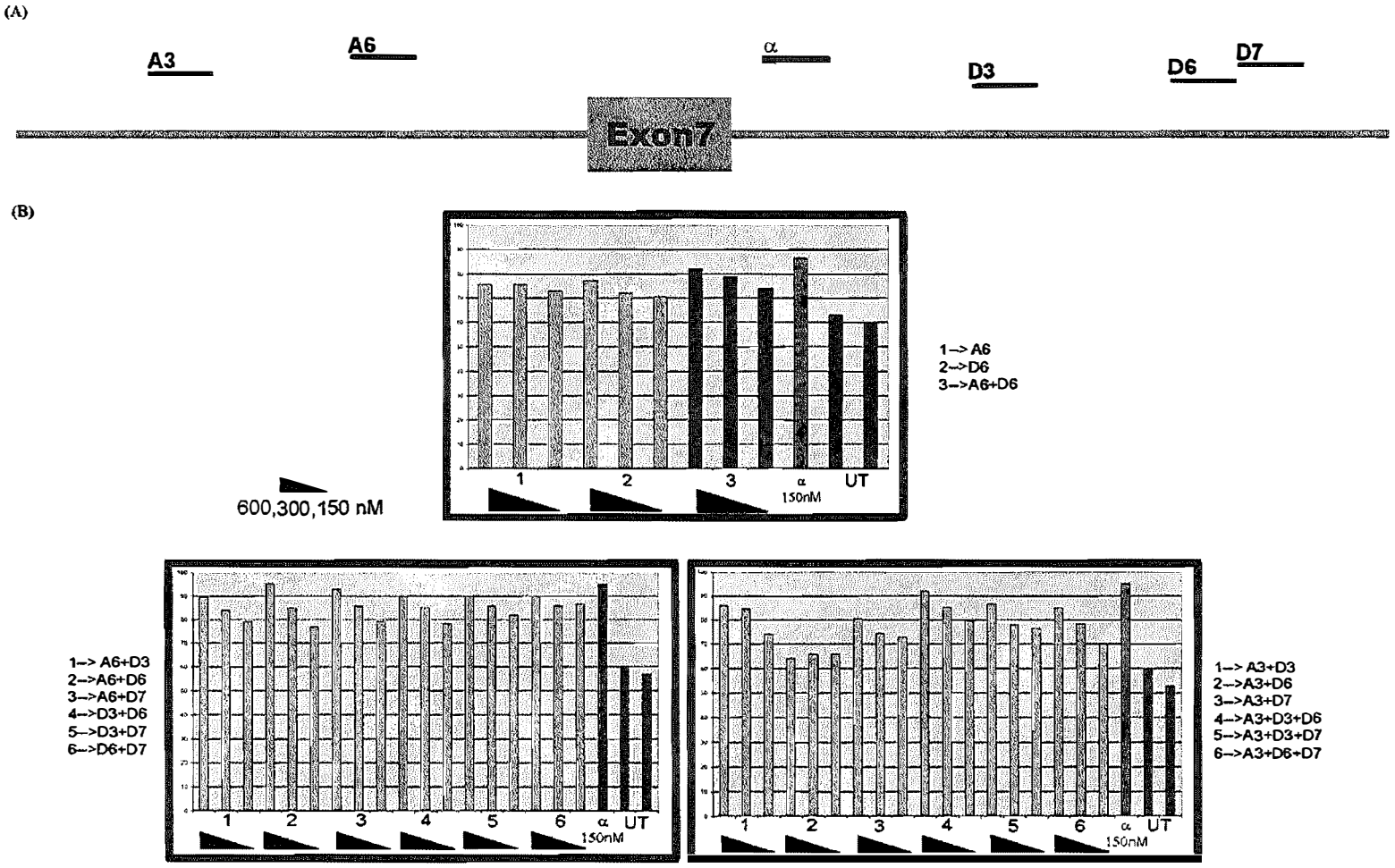

Fig 7: Efficiency of AO-cocktails containing different oligomers to induce exon 7 inclusion.

(A) Relative annealing coordinates of five oligomers selected on the grounds of their efficiency in causing exon 7 inclusion. (B) Levels of full-length SMN1 and SMN2 transcripts containing exon 7, as determined by densitrometric analysis of RT-PCR of RNA from cultured fibroblasts from an SMA patient treated with twelve AO-cocktails targeting introns 6 and 7 at different concentrations (150, 300 and 600nM), relative to the levels of the two major isoforms (exons 4+5+6+7+8 and exons 4+5+6+8).

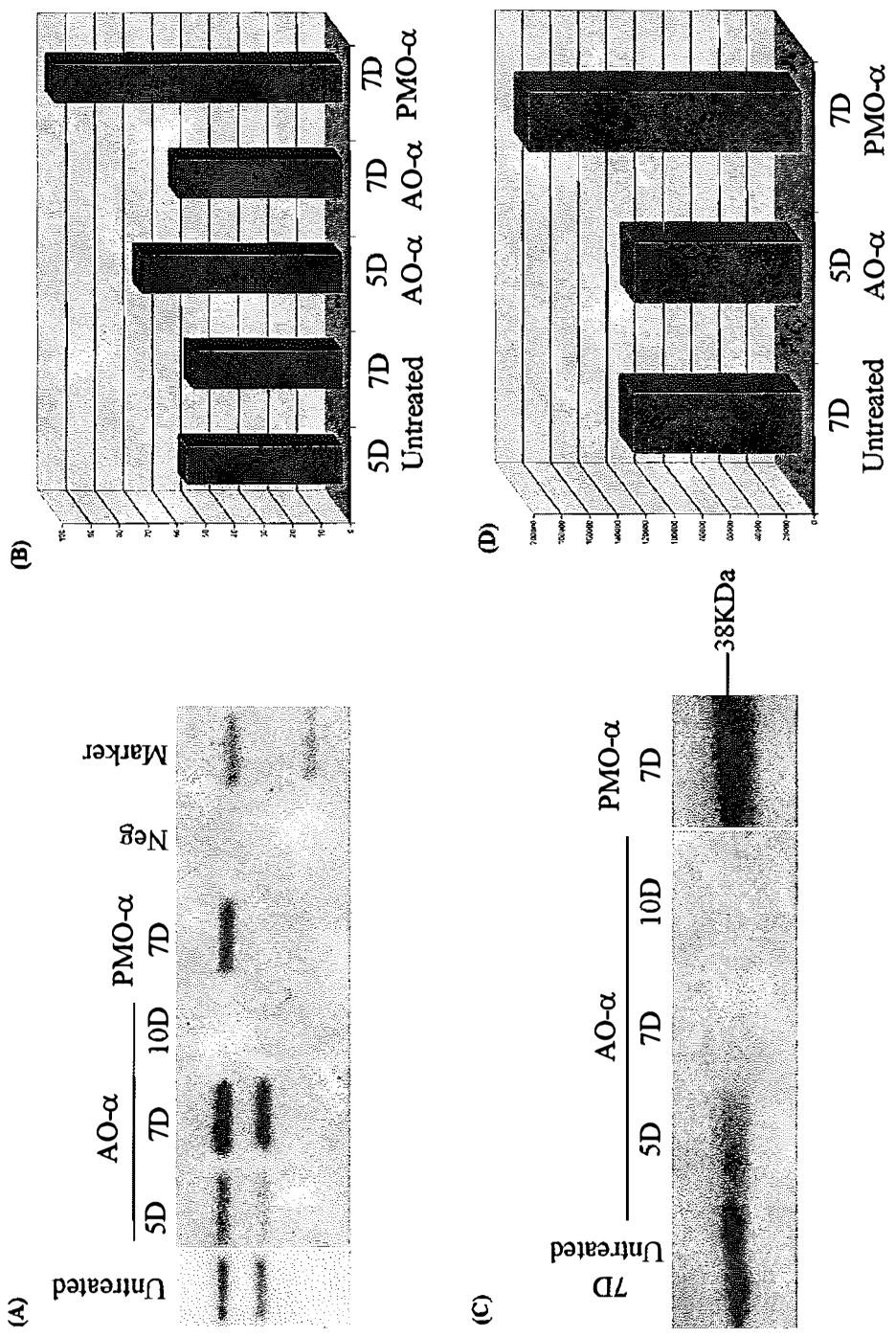

Fig 8: Efficiency of oligomers with different backbone chemistries in inducing SMN expression.

(A) RT-PCR and (B) densitometric analysis of full-length SMN1 and SMN2 transcripts containing exon 7 in cultured fibroblasts from a SMA patient at different times (5, 7 or 10 days) after treatment with either 2'-O-methyl modified oligomer α on a phosphorothioate backbone (AO-α) or phosphorodiamidate morpholino oligomer α (PMO-α), relative to levels of the two major isoforms (exons 4+5+6+7+8 and exons 4+5+6+8). (C) Western blots using anti-SMN antibody and (D) densitometric analysis of SMN protein from cultural fibroblasts from a SMA patient treated with AO-α or PMO-α for 5, 7 or 10 days, compared to untreated fibroblasts.

Table 1: Size, annealing coordinates and sequences of oligomers used in this study.

AOs	size	coordinates	sequences	GC content		melting
				(bp)	%	temperature
α	20	SMN2E7D(-10-29)	5' AUU CAC UUU CAU AAU GCU GG 3'	7	35	54
b	22	SMN2E7D(-10-31)	5' AGA UUC ACU UUC AUA AUG CUG G 3'	8	36	60
c	22	SMN2E7D(-12-33)	5' UAA GAU UCA CUU UCA UAA UGC U 3'	6	27	56
d	22	SMN2E7D(-14-35)	5' AGU AAG AUU CAC UUU CAU AAU G 3'	6	27	56
e	22	SMN2E7D(-16-37)	5' AAA GUA AGA UUC ACU UUC AUA A 3'	5	23	54
f	15	HSMN2A(+7+21)	5' UUU UUG AUU UUG UCU 3'	3	20	36
g	15	HSMN2A(+34+48)	5' AUU UAA GGA AUG UGA 3'	4	27	38
A1	20	HSMN2A-170-151	5' AUA GUC UUU UAA UGU ACU UU 3'	4	20	48
A2	20	HSMN2A-150-131	5' UAU GAU CAG AAA UUA AGU UG 3'	5	25	50
A3	20	HSMN2A-130-111	5' ACU UAU UUU AUU CAA CAA AA 3'	3	15	46
A4	20	HSMN2A-110-91	5' UUU GUU UCA CAA GAC AUU UU 3'	5	25	50
A5	20	HSMN2A-90-71	5' AUA UGG AUG UUA AAA AGC AU 3'	5	25	50
A6	20	HSMN2A-70-51	5' AGC UAU AUA UAG AUA GCU UU 3'	5	25	50
A7	20	HSMN2A-50-31	5' AUA GCU AUA UAG AUA UAG AU 3'	4	20	48
A8	20	HSMN2A-30-11	5' AAU AAA GGA AGU UAA AAA AA 3'	3	15	46
A9	30	HSMN2A-80-51	5' AGC UAU AUA UAG AUA GCU UUA UAU GGA UGU 3'	8	27	76
A10	25	HSMN2A-75-51	5' AGC UAU AUA UAG AUA GCU UUA UAU G	6	24	62
A11	25	HSMN2A-90-66	5' GCU UUA UAU GGA UGU UAA AAA GCA U 3'	7	28	64
A12	25	HSMN2A-82+58	5' UAU AGA UAG CUU UAU AUG GAU GUU A 3'	6	24	62
D1	20	HSMN2D-35-54	5' AAC CAU AAA GUU UUA CAA AA 3'	3	15	46
D2	20	HSMN2D-55-74	5' AAA AAC AUU UGU UUU CCA CA 3'	5	25	50
D3	20	HSMN2D-75-94	5' UCU GAA CUU UUU AAA UGU UC 3'	5	25	50
D4	20	HSMN2D-95-114	5' AAC CUU UCA ACU UUC UAA CA 3'	6	30	52
D5	20	HSMN2D-115-134	5' AAU AUU GAU UGU UUU ACA UU 3'	3	15	46
D6	20	HSMN2D-135-154	5' UUU UGG CAU CAA AAU UCU UU 3'	5	25	50
D7	20	HSMN2D(-155-174)	5' AUU AAC CUU UUA UCU AAU AG 3'	4	20	48
D8	20	HSMN2D(-175-194)	5' GAA UUC UAG UAG GGA UGU AG 3'	8	40	56
D9	25	HSMN2D(-70-94)	5' UCU GAA CUU UUU AAA UGU UCA AAA A 3'	5	20	60
D10	25	HSMN2D(-75-99)	5' UAA CAU CUG AAC UUU UUA AAU GUU C3'	6	24	62
D11	25	HSMN2D(-130-154)	5' UUU UGG CAU CAA AAU UCU UUA AUA U 3'	5	20	60
D12	25	HSMN2D(-135-159)	5' AAU AGU UUU GGC AUC AAA AUU CUU U 3'	6	24	62
D13	25	HSMN2D(-150-174)	5' AUU AAC CUU UUA UCU AAU AGU UUG 3'	5	20	60
D14	30	HSMN2D(-145-174)	5' AUU AAC CUU UUA UCU AAU AGU UUU GGC AUC 3'	8	27	76

Chapter 7

Conclusions and implications for future research

There has been enormous progress in the field of antisense applications with improved methods for oligonucleotide production, leading to consistent and reliable use of antisense oligomers (AOs) against a number of gene targets, through various mechanisms (viz. RNaseH-dependent gene knockdown, interfering splicing motifs, mRNA translation modulation and miRNA knockdown). Typically, DNA analogues were utilised to anneal to mRNA, thereby recruiting and activating RNaseH-dependent mRNA degradation of the target.[1] The use of new AO chemistries of 2′-O-methyl modified sugars (2OMe) or 2′-O-methoxy ethyl modified sugars (MOE) on phosphorothioate backbones, peptide nucleic acids (PNAs), locked nucleic acids (LNAs) and phosphorodiamidate morpholino oligomers (PMOs), which all confer greater stability and affinity to an mRNA target, have created new generation AOs able to modify gene expression by mechanisms other than RNaseH-dependent mRNA transcript degradation.[2]

In fact, control of gene expression through RNA oligomers is a natural process. Surprisingly, 93% of investigated genomic bases are transcribed in different human cells.[3] Noncoding RNAs (ncRNAs) account for 50 to 75% of all transcripts in higher eukaryotes.[4, 5] Although the majority of documented ncRNAs have not been studied in their biological roles, there is some evidence that most steps of gene expression, including transcription,[6] splicing[7] and translation,[8] are controlled by natural antisense transcripts.

microRNA (miRNAs) are approximately 22-nucleotide-long untranslated RNAs, and act post-transcriptionally to control gene expression.[9-11] Imperfect base pairing between the seed region (two to eight nucleotides) of an miRNA and target transcripts, which share identical seed regions (**Fig 1**), allow an miRNA to repress translation in a number of genes, which possibly participate in common cellular processes. Chemically modified oligomer analogues are unable to induce miRNA effects on target genes as these synthetic compounds do not form a complex with the argonaute protein (AGO). Nevertheless, a novel AO-based intervention has been developed, where 'antagomirs', sequences complementary to the miRNA, have been utilised as decoys to intercept binding between an miRNA and the target RNA.[12]

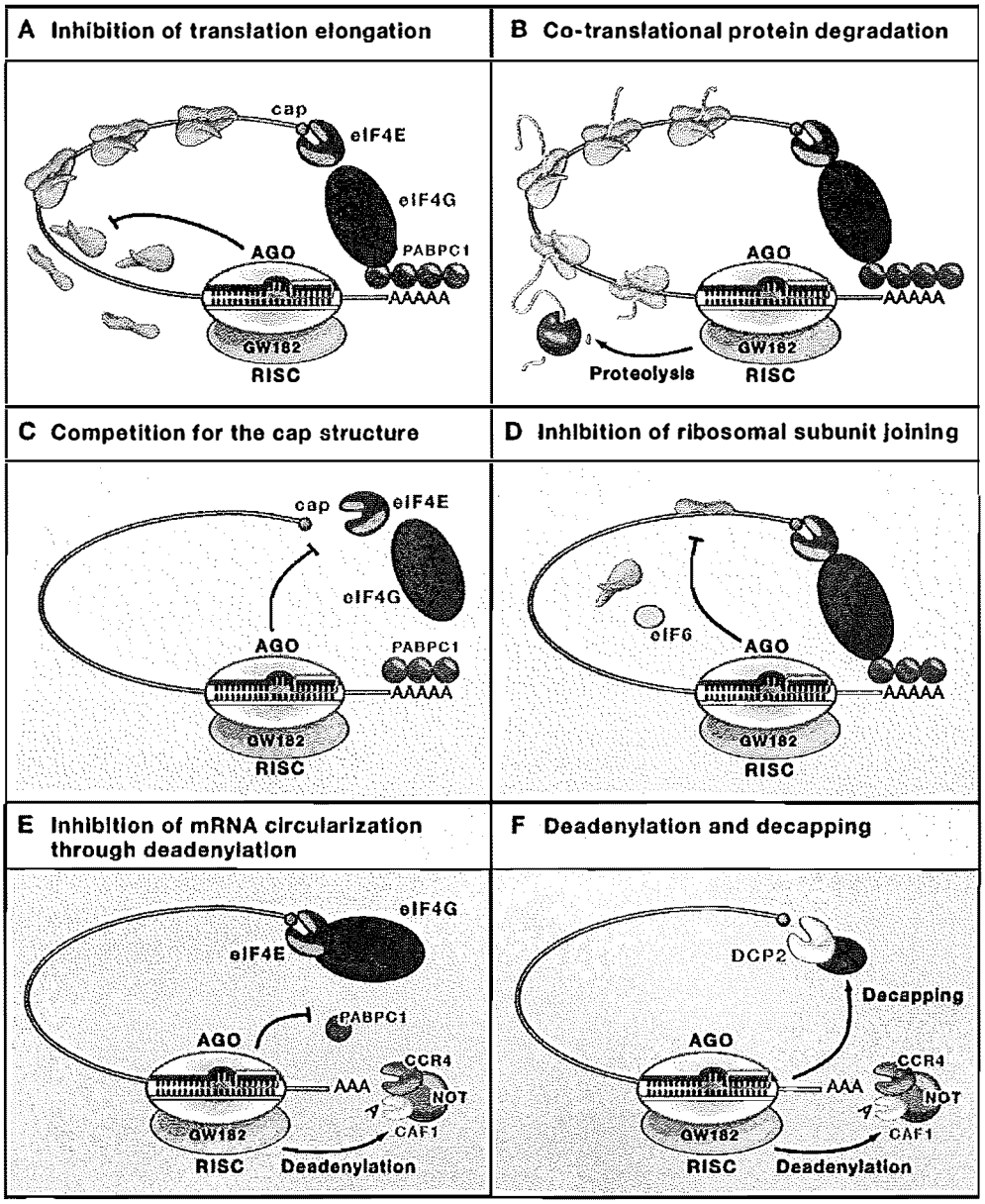

Figure 1: Possible mechanisms of miRNA-mediated gene silencing.

(A) miRNAs (dark red) inhibit targeted mRNA translation by promoting premature dissociation of ribosomes; thereby impeding translation elongation; (B) miRNA-associated RISC (RNA-induced silencing complex) activates proteases to cotranslationally degrade the nascent polypeptide chain; (C-E) Three possible mechanisms of miRNA interference via translation initiation complex are through a competitive inhibitory effect of Argonaute protein on the initiation complex; (F) miRNAs trigger deadenylation and subsequently decapping of target RNA. Light green rectangular boxes demonstrate miRNA seed, seven to eight nucleotides at the 5' end of an miRNA, which determine miRNA specificity to target mRNA. (Modified from Eulalio *et al.* 2008[11])

Another possibility to modulate gene expression at a different level is the use of AOs to induce a particular splice isoform. Pioneering research on a β-globin artificial construct confirmed that AOs were able to mask cryptic splice sites, thereby normalising an aberrant splicing pattern.[13] Subsequently, oligomers were used to induce exon skipping of the human dystrophin exon 19 in Ebstein-Barr virus transformed lymphoblastoid cells derived from a normal human donor.[14] The AOs were designed to target motifs predicted to be involved in exon definition, located in human dystrophin exon 19,[14] based on an intra-exonic deletion in a DMD patient that resulted in the loss the entire exon.[15] ESEs were subsequently postulated to be amenable targets for AO-induced exon skipping.[14, 16]

The AO-induced exon skipping approach has been embraced as an option for the treatment of DMD after reports of AO induction of near-normal dystrophin levels in *mdx* mice and myoblast cultures from DMD patients.[17-22] The primary feature of this thesis demonstrates an AO-based approach as a targeted treatment of another mouse model of muscular dystrophy, the 4^{CV} mouse. It was originally anticipated that this would be a relatively simple task, since exon skipping was efficiently induced in the *mdx* mouse model after targeting the exon 23 donor splice site,[22] and the equivalent human exons 52 and 53 could be removed at high efficiency.[23] However, the same motifs were not responsive in the 4^{CV} model, and it was necessary to initiate studies *de novo.*

AOs were designed to target splice motifs and ESEs in the mouse dystrophin gene as predicted by a web-based program (ESEfinder 3.0). The oligomers were then optimised based on their ability to induce exon skipping in mouse myoblasts, to identify the most active oligomer combinations. Although AO optimisation to dislodge mouse dystrophin exon 23 in the *mdx* mouse was readily achieved, induction of a transcript missing only mouse dystrophin exon 53 proved to be more challenging. The majority of individual AO treatments to remove mouse dystrophin exon 53 were ineffective. Some resulted in cryptic splice site activation, while others led to non-specific, out-of-frame and hence undesirable transcripts missing exons 53+54. Only the use of an AO-cocktail could induce a BMD-like in-frame transcript missing exons 52+53, albeit at relatively low levels of expression. Further refinement of the AO target by masking different splicing motifs resulted in more substantial levels of exons 52+53 skipping, leading to a dramatic four-fold enhancement of dystrophin expression compared to the original cocktail.

Theoretically, AO-induced exon skipping could be of benefit in restoring the dystrophin reading frame in up to 90% of DMD patients.[24] However, individual responses to the treatment of each different mutation are likely to vary, depending upon the nature and size of the gene lesion. Although, treatment with AOs to induce exon 51 skipping should be amenable to DMD patients with deletions of exons 19 to 50, exons 35 to 50 or exon 50 only, the induced dystrophin isoforms in these three examples would not restore muscle functionality to a similar extent, due to substantial differences in the restored proteins. The clinical benefit to DMD patients, whose mutations impinge upon regions integral to the function of dystrophin, will also be minimal.

Knowledge of the extent of improvement in muscle functionality, expected from an induced in-frame transcript could indicate which DMD mutations might be more amenable to AO-induced exon skipping. One possible way forward would be to combine AOs targeting different mouse dystrophin exons into cocktails, and undertake *in vivo* evaluation of mice transiently expressing induced dystrophin isoforms. Mice treated with different AO-cocktails could allow us to investigate both levels of dystrophin expression and muscle functionality. The use of AO cocktails, as undertaken in this thesis to make dystrophin isoforms, could provide the basis for predicting functional consequences of dystrophin isoforms, and postulate which DMD mutations in humans are likely to be benefit from such an approach. Such a model will be of particular benefit in designing strategies to by-pass mutations downstream of exon 62, where very few BMD-causing mutations have been reported. This strategy is based upon assumption that the human and mouse dystrophins have similar functionality.

In addition, the use of cocktails should allow a standardised treatment for clustered mutations. For example, induction of a consistent isoform (Δ45-55), which may have a uniform function in people with different mutations, could benefit up to 63% of DMD with a deletion.[25] As a single cocktail, regulatory authorities may regard this preparation as a single drug which may, in turn, expedite the implementation of a treatment to many DMD boys.

However, removal of eleven consecutive exons (exons 45 to 55) is technically challenging at this time. Individual targeted exons could be thoroughly optimised to obtain highly efficient AOs in inducing single exon skipping, however, that does not guarantee robust multi-exon skipping. Combination of optimised AOs, in some cases, did not induce efficient multi-exon skipping, presumably because of interactions

between the AO-sequences, or different efficiency in exon removal (Adams, AM unpublished observations).

To date, there are two Phase I clinical trials using oligomers to induce exon 51 skipping and restore dystrophin protein in amenable DMD patients. In the Leiden trial, four 8 to 12 year old DMD patients were injected into tibialis anterior muscles with a 2OMeAO (PRO051), which was designed to induce exon 51 skipping.[26] The Leiden trial was completed and dystrophin restoration was reported in all 4 patients. A dose escalating study is currently underway in the United Kingdom with a PMO (AVI-4658), also inducing exon 51 excision. These initial two clinical trials will only provide proof-of-principle of AO-induced exon skipping in DMD patients. However, since relatively low doses of oligomer of two different chemistries were administered by intramuscular injection, only limited information on safety can be generated. Systemic trials with PRO051 are underway, as body-wide dystrophin expression must be addressed.

AOs, in theory, can be applied to many conditions modulating the level of gene expression, through a multiplicity of processes. These processes are, indeed, combinatorially controlled and form a regulatory network governing global gene expression. On a cautionary note, we need to be aware that AOs may modify gene expression through more than one mechanism and may indeed modify the expression of other genes. As a consequence, the benefits of an antisense therapy targeted to a specific mutation need to be balanced against possible non-antisense or non-specific effects, caused by interaction of AOs with other regulatory processes and other genes, making it necessary to weigh risk against benefit, before considering their use in the treatment of patients. Assessing risk using transcriptomic and proteomic analysis on treated cultures or animals may ensure that antisense therapy not only leads to the theoretically predicted transcripts but also to the anticipated global changes in RNA and protein expression in response to synthesis of a functional protein. One way to minimise possible adverse-effects is to design the most effective compounds so that only low dosages need to be applied. Alternatively, if some particular sequences were found to cause off-target effects, other AOs targeting different motifs could be used instead.

In addition to exon skipping, the use of antisense modulation of pre-mRNA splicing may potentially target splice silencing motifs to promote exon inclusion. AOs may provide steric hindrance, which prevents the spliceosome recognising splice silencing motifs, thereby enhancing incorporation of the exon into the mature transcript.

In the second part of this thesis, this rationale was applied in the design of an array of oligomers to induce exon 7 inclusion into the human SMN2 gene transcripts in cultured fibroblasts from an SMA patient. The goal of the study was to develop protocols to identify oligomers capable of inducing exon inclusion, which may potentially treat SMA. Since the majority of human gene transcripts are alternatively processed,[27] the ability to selectively incorporate or excise an exon could be relevant to a number of other genetic disorders.

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Personalised Genetic Intervention for Duchenne Muscular Dystrophy: Antisense Oligomers and Exon Skipping

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Abstract: Duchenne muscular dystrophy (DMD) arises from protein-truncating mutations in the large dystrophin gene that preclude synthesis of a functional protein that primarily stabilizes muscle fibre membranes. The absence of dystrophin leads to this most common and serious form of childhood muscle-wasting. Since the identification of the dystrophin gene in 1987, cell and gene repair or replacement therapies have been evaluated for DMD treatment and one genetic intervention, exon skipping, is now in clinical trials. Antisense oligomers have been designed to redirect dystrophin splicing patterns so that targeted exons may be removed from a defective dystrophin pre-mRNA to either restore the reading frame of a deletion, or excise an in-frame exon corrupted by a nonsense mutation or micro-insertion/deletion. This review discusses the evolution of oligomer induced exon skipping, including *in vitro* applications, evaluation of different oligomer chemistries, the treatment of animal models and alternative exon skipping strategies involving viral expression cassettes and ex vivo manipulation of stem cells. The discussion culminates with the current clinical trials and the great challenges that lie ahead. The major obstacle to the implementation of personalised genetic treatments to address the many different mutations that can lead to DMD, are considered to be establishing effective delivery regimens for the different patients and their mutations. Furthermore, the view of regulatory authorities in assessing preclinical data on potentially scores of different but class-specific compounds will be of paramount importance in expediting the clinical application of exon skipping therapy for this serious and relentlessly progressive muscle wasting disease.

Keywords: Antisense Oligonucleotides, Exon skipping, Duchenne muscular dystrophy, Morpholino, clinical trials, dystrophin, personalized medicine, pre-mRNA splicing.

INTRODUCTION

When considered individually, serious monogenic disorders are fortunately relatively rare, especially when compared to complex traits such as Alzheimer's, asthma, diabetes, or acquired conditions, including pathogenic infections and cancer. The most common human autosomal recessive disorder, cystic fibrosis, is reported to occur at a frequency of about 1 in 2100 in the Caucasian population [1]. However, when viewed collectively, single gene disorders are an enormous burden to those affected, their families, communities, and the health care system.

It is no longer appropriate to consider single gene disorders as "simple", particularly since it is apparent that different lesions in a particular gene can result in a variety of clinically distinguishable conditions, with either recessive or dominant modes of transmission. Although the ΔF508 mutation defect accounts for about 75% of cystic fibrosis cases [2, 3], over 1,000 different mutations have been reported and the clinical presentation can vary considerably from severe, with extensive lung involvement, to a very mild phenotype with reduced fertility as the predominant symptom. Similarly, different mutations in the huge dystrophin gene can lead to the allelic conditions, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), and X-linked dilated cardiomyopathy [4].

Restoration of expression of a single component to address a missing or defective gene product is the basis of gene replacement (viral and non-viral systems) or cell-based therapies. Although great advances are being made in the treatment of some conditions, for example, gene replacement in children with X-linked severe combined immune deficiency (SCID) [5, 6], there have also been serious adverse events and progress has not been as rapid as anticipated [7]. Challenges to gene and cell replacement therapy for DMD arise from the size of the gene product and/or nature of the gene expression. Dystrophin, the affected gene product in DMD, is encoded by the largest known gene and is expressed as multiple isoforms in different tissues, with the 427 kDa skeletal muscle protein produced at low amounts in about 30% of the human body mass [8].

Such challenges in gene and cell replacement for DMD have prompted the examination of other possible therapeutic interventions, including up-regulation of a homologous protein or redirecting expression, processing, or translation of the defective gene product [9-11]. This review will consider the use of antisense oligomers (AOs) to reduce the severity of DMD, through specific redirection of dystrophin pre-mRNA processing to by-pass protein-truncating mutations during mRNA maturation. The nature of this genetic therapy is such that specific AOs and exon skipping strategies must be tailored to address different mutations. Although this may sound daunting, development of personalized treatments should not be considered unrealistic. Many compounds have now been optimised to address different dystrophin mutations *in vitro*, and two compounds are currently in clinical testing to restore the reading frame of a common type of dystrophin mutation. Demonstration of dystrophin expression in these trials should facilitate implementation of other AO strategies for different DMD patients, particularly if exon skipping could be regarded as a generic therapy.

DUCHENNE AND BECKER MUSCULAR DYSTROPHY

DMD and BMD are allelic X-linked recessive conditions arising from mutations in the dystrophin gene. One third of cases are *de novo* and germ-line mosaicism has been reported to be as high as 14% [12]. The tremendous size of the dystrophin gene and organization of repeated elements within some introns in excess of 200kB, are thought to contribute to the high spontaneous mutation rate. DMD occurs at a cited incidence of 1 in 3,500 live male births, and is the most common severe muscular disorder in childhood [13]. DMD individuals appear normal at birth, but present with muscle weakness between the ages of 3-5 years. Muscle degeneration gradually overwhelms regenerative capacity in a relentlessly progressive and predictable manner. As muscle degeneration progresses, affected individuals exhibit difficulties arising from the

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1874-4672/09 $55.00+.00

Table 1. Some Examples of BMD Deletions with Associated Comments on Phenotype

Exonic Deletion	Special comments	Reference
3-9	Playing competitive badminton at age 62 years.	[16]
9-22	High CK, myalgia but well developed musculature and no evidence of muscle weakness	[134]
13-18	Myalgia and cramps after normal activity	[135]
13-41	Very mild BMD	[136]
17-47	Source of the dystrophin mini-gene used in gene replacement studies	[34]
17-51	Mild BMD with congenital cataracts	[137]
35-44	Cramping after soccer or mountain climbing	[138]
41-44	Elevated CK, otherwise asymptomatic	[139]
45-53	Diagnosed age 60	[140]
48	Accidentally diagnosed in female, four affected male members then diagnosed with high CK only.	[141]
50-53	Elevated CK, otherwise asymptomatic	[139]
It should be noted that very few BMD patients have been identified with in-frame deletion in the central rod domain involving exons 33-45.		[142]

floor, climbing stairs and running, and eventually lose ambulation before the age of 12 years. The most common causes of death are cardiac or respiratory complications, but improvements in heath care, the use of steroids and assisted ventilation have extended the life span of DMD patients by up to 50% over the last two decades [14].

BMD is estimated to occur at one tenth the frequency of DMD [15]. Presenting with a spectrum of severities, BMD is clinically classified as an individual becoming wheelchair bound by age 16 years or later, while some are apparently asymptomatic and may only be diagnosed later in life [16]. Examples of gross dystrophin gene deletions that have been identified in mildly affected BMD patients are shown in Table 1, where it can be seen that over 50 dystrophin exons may be deleted, in various combinations, with relatively minor clinical consequences. It seems probable that the low incidence of BMD, compared to DMD, may be due to the inability to identify cases that do not present with overt symptoms. A mutation in the dystrophin gene was recently reported where the loss of exon 16 did not raise the serum creatine kinase levels [17], a sensitive marker of muscle damage.

The molecular distinction between DMD and BMD depends upon the quantity and quality of dystrophin that can be synthesized as a consequence of the gene lesion [18]. Genomic deletions of one or more exons, found to cluster in 2 hotspots near huge introns, are the most common type of dystrophin mutation and account for about 60% of cases. Duplications, typically involving multiple exons are generally found in the proximal third of the gene and are seen in 8-15% of DMD cases [19-22]. Disruption of the reading frame that leads to premature termination of dystrophin synthesis results in DMD, while in-frame gene re-arrangements typically allow the generation of internally truncated dystrophin that retains some biological function. The variable manifestation of BMD, from border-line DMD to asymptomatic, reflects the quality and/or quantity of the internally shortened dystrophin (for review see [23-25]).

The dystrophin gene contains 79 exons spanning approximately 2.4 million base pairs and encodes 3 major isoforms, which are expressed primarily in muscle, heart and brain. The reading frame organization of the 79 exons is shown in Fig. (1). In addition, there are 4 internal promoters, which encode shorter isoforms expressed in other tissues. Due to the catastrophic consequences of dystrophin loss on muscle function and integrity, it is presumed that the 427 kDa skeletal muscle isoform is most important, hence this has been the focus of the gene repair or replacement studies (for review see [26, 27]).

Dystrophin is thought to act much like a shock absorber linking the actin cytoskeleton to a complex of proteins embedded in the sarcolemma. The primary actin binding domain involves dystrophin exons 2-8, although secondary actin binding sites have been identified [28, 29], supported by the identification of mildly affected BMD individuals missing exons 3-9 [16]. Dystrophin exons 62-69 encode the cysteine rich domain, which is pivotal in the linkage of dystrophin to β-dystroglycan, and hence to the dystrophin associated proteins and glycoproteins embedded in the sarcolemma. In addition to the primary structural role linking the actin cytoskeleton and the sarcolemma, dystrophin also anchors syntrophin, dystrobrevin, and localizes nNOS [25]. As well as the major 427 kDa isoforms expressed in muscle, heart and brain, internal promoters located within introns 30, 45, 56 and 63 are responsible for the production of the shorter isoforms, DP260, DP116, DP140, and DP71, respectively. In non-muscle tissue, DP116 associates with the sarcoglycan complex and is implicated in myelin stability in schwann cells [30]. Transient expression of DP140 in embryonic kidney is involved in renal tubulogenesis [31]. The carboxy (C)-terminal domain of DP71 isoforms plays an important role in neuronal differentiation [32].

The actin and β-dystroglycan binding domains are separated by 24 spectrin-like repeats, and this rod domain is encoded by about two thirds of the dystrophin gene transcript [33]. Portions of the rod domain appear to be somewhat redundant in that substantial in-frame deletions of rod domain generally lead only to a mild BMD phenotype [34] (Table 1).

NATURAL PRECEDENTS FOR EXON EXCLUSION DURING DYSTROPHIN PROCESSING

There are two precedents to support the hypothesis that induced exon skipping could minimise the consequences of protein-truncating mutations in the dystrophin gene. Revertant fibres are dystrophin-positive fibres that occur naturally and have been detected in *mdx* mouse model of muscular dystrophy [35, 36], the dystrophin-deficient muscular dystrophy canine model [37-39] and at least 60% of all DMD cases [40-43]. Another canine model, a German short-haired pointer [44], in which the entire dystrophin gene was missing, did not contain any revertant fibres, suggesting that the mechanism responsible for restoring some dystrophin expression required the retention of sufficient coding sequences of the dystrophin gene.

Although revertant fibres are regularly detected in many dystrophic tissues, the frequency is insufficient to be of any substantial clinical benefit [42]. RNA analysis and epitope mapping indicated the presence of multiple revertant dystrophin isoforms in human and mouse muscle. This suggested some natural exon skipping event led to re-arrangements [36, 45], generally excluding 20 or

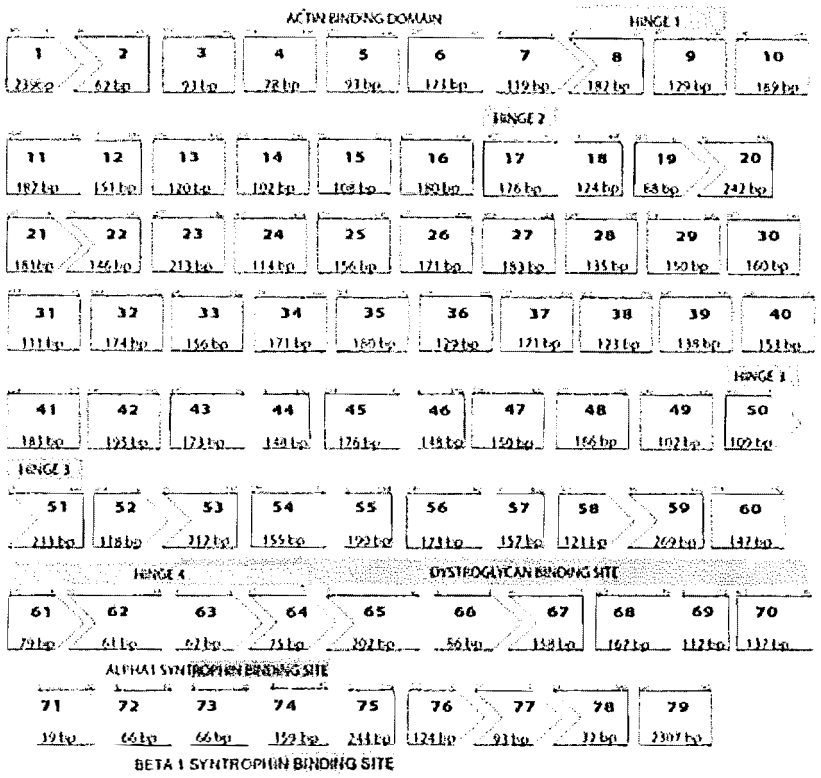

Fig. (1). Structure of the dystrophin gene transcript indicating the reading frame and major functional domains. Boxes represent in-frame exons whereas interlocking forward and reverse arrows and notches indicate codons spanning the exon:exon junction. Junction codon sequences are shown above the exons.

more exons [46]. *In situ* hybridization studies in the *mdx* mouse using exon 23 and flanking intronic sequences as a genomic probe, indicated that the dystrophin gene in the majority of revertant fibres was structurally intact, thereby excluding secondary somatic deletions in the dystrophin gene as the primary possible mechanism [46]. The underlying basis of revertant fibres is believed to involve some form of alternative splicing [36, 47], although it is difficult to conceptualize a mechanism whereby 20 exons spanning hundreds of kilobases of pre-mRNA are consistently excluded in one muscle fibre and not another.

BMD patients typically have genomic deletions in the dystrophin gene that do not disrupt the reading frame. A few point mutations that lead to BMD have been identified, including missense mutations in the actin binding domain [15] and splice motif mutations that either lead to excision of an in-frame exon, or reduce the amount of normal dystrophin mRNA [48]. Some nonsense mutations in the dystrophin gene have also been found to lead to BMD [49, 50], in apparent conflict with the reading frame hypothesis, which predicts that protein-truncating mutations should result in DMD [51]. However in these milder than expected BMD cases, it was found that the nonsense mutation influenced splicing patterns such that natural exon skipping excluded the exon containing the nonsense mutations [50]. Comprehensive screening of genomic DNA has been reported to confirm diagnosis of over 96% of DMD cases, whereas only 60% of BMD mutations were identified [52, 53]. Analysis and sequencing of dystrophin cDNA from muscle is often essential to identify the consequences of less obvious gene mutations, where a nonsense or missense mutation/polymorphism may lead to exon skipping or activation of a cryptic splice site. Similarly, deep intronic DNA changes, sometimes kilobases away from the nearest dystrophin exon, can lead to the incorporation of pseudo-exons in the mature gene transcript [54].

ANTISENSE OLIGOMERS AND MODES OF ACTION

An oligodeoxyribonucleotide was reported to inhibit Rous sarcoma virus replication in cell culture nearly three decades ago [55, 56]. Antisense technologies became synonymous with gene down-regulation studies, most commonly through the induction of RNAseH degradation of the RNA strand in the AO:RNA duplex. Inadequate controls confounded results of some early experiments, where apparent gene suppression arose from non-sequence specific effects, in particular the sequestration of transcription/translation proteins by the phosphorothioate backbone. Despite these early setbacks, which saw antisense technology regarded with a great deal of skepticism, there has since been great progress in terms of new chemistry development and identifying distinct mechanisms of altering gene expression.

New oligomer chemistries, with modified bases and/or backbones, generated compounds with greatly increased annealing affinity and/or enhanced resistance to nuclease degradation. Vitravene (fomivirsen) was the first antisense drug to achieve marketing clearance in the USA to treat cytomegalovirus retinitis in people with AIDS [57]. In addition, new oligomer chemistries were able to influence gene expression through mechanisms other than RNaseH-induced degradation. Chemically synthesised RNA oligomers are routinely used in gene silencing studies in nematodes, plants and human cells [58-61]. Some second or third generation oligomers, which were not able to activate silencing pathways, can modify gene expression at different stages. Depending upon the target sequence design, some modified oligomers can suppress translation by masking motifs essential for ribosomal initiation and elongation, while other oligomers can anneal to motifs involved in exon recognition and intron removal, thereby redirecting pre-mRNA processing.

EARLY SPLICE INTERVENTION STUDIES

Kole and colleagues first reported the use of AOs to correct aberrant β-globin pre-mRNA splicing fifteen years ago [62]. Intronic mutations in this gene activated cryptic splice sites and account for nearly 30% of β-thalassemia cases. Despite the presence of intact splice donor and acceptor sites, the selection of cryptic splice sites led to some intron retention in the mutant β-globin mRNA. AOs consisting of 2'-O-methyl modified bases on a phosphorothioate backbone (2OMe) were designed to anneal to the cryptic splice sites, rendering them inaccessible to the splicing machinery, which in turn defaulted to the selection of the normal splice sites. A few years later, Matsuo and colleagues were able to induce an abnormal dystrophin gene transcript by targeting an oligodeoxynucleotide on a phosphorothioate backbone (ODN) to an internal domain within dystrophin exon 19 [63]. The "Kobe" DMD mutation was found to arise from a 52 bp deletion within dystrophin exon 19 that, while leaving the donor and acceptor sites intact, resulted in the excision of the entire exon from the gene transcript [64, 65]. Matsuo and colleagues hypothesized that the small intra-exonic deletion removed crucial exon recognition motifs and resulted in complete exon removal. An AO was designed to induce dystrophin exon 19 skipping in normal lymphoblastoid cells transformed with Epstein-Barr virus [63]. Hence, the first demonstration of exon skipping in the dystrophin gene did not rescue defective gene expression, but induced abnormal splicing and disrupted the reading frame. Nevertheless these experiments confirmed the principle of targeted exon skipping and proposed this approach as a potential therapy for DMD.

ANIMAL MODELS

The most commonly studied animal models of dystrophin mutations are the *mdx* mouse, which carries a nonsense mutation in exon 23 [35], and the Golden retriever muscular dystrophy model (GRMD), which was found to have a splice site mutation that leads to exon 7 exclusion and a subsequent mRNA frame-shift [39]. The *mdx* mouse model is readily available, inexpensive, but suffers from the limitation that the dystrophin deficiency does not induce an overt severe dystrophic phenotype. Nevertheless, detailed examinations show raised serum creatine kinase levels, muscle weakness and some muscles, in particular the diaphragm, show extensive fibrosis and dystrophic pathology [66]. As these animals age, the consequences of the dystrophinopathy become more obvious, but these animals can voluntarily run in a manner similar to wild type animals. Another mouse model, which does show a very severe phenotype is the utrophin/dystrophin double knockout mouse [67], although no exon skipping studies in this model have been reported to date.

The canine GRMD model is much more severely affected, perhaps more so than the human condition, and provides a more relevant model in which to assess any therapies. Limitations of the canine model include substantial variation in severity, excessive costs involved in maintenance and care, limited numbers available from each litter, and the emotive issues associated with working on companion animals.

AO DESIGN AND TARGET SELECTION

The rescue of dystrophin expression using AO-induced exon skipping in the *mdx* mouse was first reported by Dunkley and colleagues, who described a 2OMe AO 12mer directed at the exon 23 acceptor splice site that generated a transcript in which exons 23 to 29 had been skipped. 2OMe AOs directed at the branch point or the donor splice site of exon 23 were not found to have any effect on the dystrophin mRNA processing [68]. In contrast, the following year we demonstrated that targeting the exon 23 donor splice site with a 2OMe AO 20mer was able to induce precise and consistent exon 23 skipping, whereas a 20mer directed at the acceptor site was found to be ineffective [69]. Subsequent refinements in AOs targeting the dystrophin exon 23 donor splice site were reported and found to further enhance the capacity of the AO to induce exon 23 skipping [70]. Additional AO refinement at the acceptor splice site consistently failed to induce any detectable skipping [70].

Studies describing the application of AOs directed to exons flanking frame-shifting genomic deletions in DMD patient cell lines were reported by van Deutekom and colleagues [71]. The Leiden muscular dystrophy database (http://www.dmd.nl/) list exon 45 as one of the most commonly deleted exons in DMD, whereas the in-frame deletion of exons 45 and 46 is associated with a mild form of BMD. van Deutekom and colleagues designed a 2OMe AO to motifs within exon 46 and conclusively demonstrated restoration of the reading frame in cells from DMD individuals carrying a dystrophin genomic deletion of exon 45 [71]. These studies were then extended to a variety of other mutations in the dystrophin gene [72-74].

It has been proposed that only 12 different AOs would be able to restore the reading frame in the majority of DMD deletion patients, particularly since this type of mutation is clustered in the minor and major dystrophin deletion hotspots [75]. Indeed, the most commonly deleted exons lie between exons 45 and 55, and Beroud and colleagues [76] reported that multiple exon skipping across those exons could restore some functional dystrophin expression in almost two thirds of all DMD patients.

However, more than one-third of DMD cases do not arise from genomic deletions and these patients should not be excluded from any potential exon skipping therapy. Nonsense mutations, splicing defects and micro-insertion/deletions have the potential to lead to premature termination of translation, and these defects appear evenly distributed across the entire gene. As discussed previously, an apparent catastrophic DNA change such as a nonsense mutation, does not necessarily lead to premature termination of translation, if the DNA variant compromises exon recognition and results in variable levels of natural exon skipping. Since the excluded exon is in-frame, a protein typical of BMD is generated [49, 50], again providing evidence that exon skipping has the potential to ameliorate DMD progression.

Furthermore, unlike many genomic deletions involving multiple exons, the entire dystrophin coding region is present in the non-deletion DMD patients. The removal of one or two exons to by-pass a protein-truncating mutation is unlikely to seriously compromise the function of the induced dystrophin, unless the exons code for a crucial functional domain. Aartsma-rus and colleagues [77] described 114 AOs that target 35 exons for removal. In 2007, we released the first draft of AOs targeting every exon in the dystrophin pre-mRNA for excision, excluding the first and last exons [78].

It has been suggested that directing AOs to dystrophin donor or acceptor splice sites may lead to off-target effects on other splice sites [79], a possibility which cannot be discounted. However, the invariant bases of the acceptor and donor splice sites are only 2 nucleotides long, and occur at the end and beginning of each intron flanking the target exon (---ag[EXON]gu---). We took the approach that any motif involved in splicing must be regarded as a potentially amenable target and evaluated the efficiency of AOs directed at acceptor and donor splice sites, as well as Exon Splicing Enhancers (ESE's) as predicted by ESEFinder [80, 81]. Although the "ag" and "gu" motifs are almost invariant at the acceptor and donor splice sites respectively, these two nucleotides would only constitute a minor proportion of the AO annealing site. It may be argued that targeting ESE's, where 6 or 8 consensus nucleotide motifs are recognised by SRp55 or SC35 respectively, offers a greater chance of cross-transcript targeting.

Remarkably from these two extensive reports on AO design to induce dystrophin exon skipping [77, 78], about two thirds of AOs designed and evaluated were able to induce some level of targeted

exon exclusion. This is consistent with the observation that many changes in the protein coding region can disrupt splicing [82], and implies that many motifs are involved in exon recognition and splicing. However, there are substantial variations in exon skipping efficiencies, as clearly some AOs targeted more amenable or responsive sites for induced exon skipping than others. Some exons were readily and efficiently removed at what was arbitrarily chosen an acceptable level *in vitro*, greater than 30% exon removal compared to the intact transcript after transfection at 100 nM AO:lipoplex [78], while other exons were more difficult to dislodge. Aartsma-Rus and colleagues [83] noted that the effective AOs targeted significantly higher numbers of SF2/ASF, SC35 and SRp40 motifs than the ineffective AOs [77].

Our strategy for AO design has been more empirically based. An initial panel of AOs were designed to target splice site junctions for each exon, as well as predicted ESE motifs. Normal myogenic cultures were transfected and the test compounds exhibiting the most pronounced exon skipping efficiency were used as a template to design a subsequent series of overlapping AOs. The most efficient AO was defined as the compound that induced maximal exon excision *in vitro*, after transfection at concentrations over the range 10 to 100 nM. Although the AOs designed to induce specific exon skipping will ultimately be applied to cells expressing a defective dystrophin gene, AO development was undertaken in normal human primary myogenic cell cultures. Designing AOs to target the normal dystrophin gene transcript places extra demands on evaluation. Unlike cells expressing a defective dystrophin mRNA subjected to increased turn-over through nonsense mediated decay [84], removal of approximately half of the exons from a normal dystrophin transcript may lead to a disruption of the reading frame. Hence, the normal gene transcript will be expressed at wild-type levels, and the induced exon deleted transcript will be subjected to faster turn-over though nonsense mediated decay. Consequently, when the appropriate therapeutic oligomer is applied to dystrophic cells, the effect on exon skipping should be more pronounced, as the reading frame will have been restored and the induced transcript no longer subjected to nonsense mediated decay (NMD). This feature was evident in evaluating AOs to excise exon 19 from the dystrophin gene transcript expressed in normal and *mdx* murine myogenic cells. The *mdx* dystrophin transcript would be subjected to NMD, as would any dystrophin transcripts missing exon 19. Despite the same nucleotide sequence and splicing machinery, exon 19 removal was induced in *mdx* cells at concentrations 4 fold lower than that required in the normal cells [85].

One trend in AO design that became evident was that the length of the AO could play a major role in determining the efficacy of induced exon skipping, although this appears to be largely dependent upon the target exon [86]. Several motifs were examined as targets for induced skipping of human dystrophin exon 16, in particular, a cluster of high-scoring potential ESE's near the donor splice site. Despite masking of high-scoring ESE's and this donor splice motif, overlapping AOs directed at the human exon 16 donor site were found to be ineffective, whereas a 25mer, spanning the acceptor site induced moderate exon 16 skipping. AOs with additional bases at the 5' or 3' end of the 25mer were found to be about four-fold more effective than the original AO targeting the acceptor. Most surprisingly, a 20mer common to all three AOs was found to be totally ineffective [86]. Although it had been observed that a 25mer directed at the mouse exon 23 donor site was marginally more effective than a 20mer, longer AOs (30mer) directed at this site were consistently found to induce less exon 23 skipping than the shorter compounds [86].

Some dystrophin exons were difficult to dislodge from the mature dystrophin mRNA, and despite designing AOs across most of the exon, with either no or only very low levels of skipping being induced after transfection with high AO:lipoplex concentrations. Exon 20 was one such example, in which over 20 different AOs were designed and evaluated, but only one compound was eventually found to induce moderate levels of exon 20 excision. Combinations of AOs were then evaluated and some, but not all AO cocktails, were found to be very effective in a clearly synergistic rather than cumulative manner [83, 87]. AO cocktail design was not as simple as combining the most effective AOs, as the optimal AO cocktail for exon 20 consisted of two compounds that had no effect on splicing when used individually. Replacing one of the AOs in this cocktail with a longer overlapping compound that did show some exon skipping potential when used alone, actually lowered the efficiency of that cocktail [87]. In another example, very weak exon 65 skipping could be induced with one AO after transfection at a concentration of 600 nM. When this AO was combined with another directed at exon 65, pronounced exon skipping was evident after transfection at a combined AO concentration of 2 nM [87].

This raises the question of exactly how AOs influence the splicing process. It had been assumed that AOs anneal to single stranded motifs on the pre-mRNA, where SR proteins or other splice factors, such as short non-coding RNAs including miRNAs [88] may be involved in exon recognition and definition. AO binding to the appropriate target would render that pre-mRNA site double-stranded and presumably prevent correct assembly of the spliceosome. If this were the case, one would assume that the more obvious motifs involved in splicing, such as the acceptor or donor sites should provide reliable targets for consistent splice intervention. This is clearly not the case, as we have identified only one human dystrophin exon in which the donor splice site was the single most amenable target for exon skipping [78]. Although directing AOs to some donor splice sites does induce exon skipping, there are more examples of no skipping whatsoever. As reported by Arechavala-Gomeza *et al* [89], applying a panel of AOs to microwalk across the donor splice site of human dystrophin exon 51, or using AOs of increased length targeting the donor site, failed to induce any substantial exon skipping. It would appear that if a donor splice motif does not appear amenable, extensive AO design and manipulation targeting that area will be a futile exercise.

Rather than directly masking motifs recognized by the various splice factors, perhaps the AOs bind to the pre-mRNA and alter secondary structures that are crucial in exon recognition and splicing. There is mounting evidence that secondary structures within the pre-mRNA are involved in both constitutive and alternative splicing [90-92] and this may account for the observations that 2 out of 3 AOs designed and evaluated were able to induce some exon skipping [77, 78]. It is possible that some donor or acceptor sites unresponsive to AO intervention are influenced by particular splicing factors, which bind very strongly and/or immediately after transcription.

Several oligomer chemistries have been identified as suitable to induce exon skipping. While ODNs were first used to induce exon 19 excision in dystrophin processing [93] and later used in the first clinical trial involving one patient [94], there are several reasons why this particular chemistry should not be taken to the clinic for induced exon skipping. This type of oligomer is more susceptible to nuclease degradation than many other chemistries and would need constant re-administration to maintain therapeutic concentration. Secondly, ODNs are typically used to induce degradation of the target gene transcript by RNaseH action. Presumably if exon excision can occur before RNaseH degradation, the induced transcripts would then be resistant to degradation, unlike the intact transcript. Although ODNs were able to induce exon 19 skipping in cultured cells, AOs of this chemistry directed at other splice motifs did not induce exon skipping [85]. Chimeric AOs consisting of a mixture of modified and unmodified bases demonstrated increased exon skipping efficiency, correlated with increased content of 2'-O-methyl modified bases [95].

One of the more commonly used nucleotide chemistry for AOs to induce exon skipping are those consisting of 2'-O-methyl modi-

fied bases on a phosphorothioate backbone [62, 69, 71, 96]. Several other AO chemistries have also been evaluated for induction of exon skipping, including terminally modified 2OMe AOs [97], 2'-O-methoxy-ethoxy AOs (MOE, unpublished data), 4'-C-ethylene bridge nucleic acids (ENAs) [72, 95, 98], locked nucleic acids (LNAs) [72, 99], peptide nucleic acids (PNAs) [72], and phosphorodiamidate morpholino oligomers (PMOs) [72, 100]. LNA, PNA, PMO, and 2OMe AOs were directly compared to remove exon 46 from a DMD cell line in which exon 45 was deleted [72]. This study found that oligomers prepared as 2OMe and LNAs could efficiently induce exon 46 removal, whereas the equivalent compounds prepared as PMOs or PNAs were ineffective [72]. These authors concluded that the 2OMe chemistry was preferable to pursue further induced exon skipping strategies [72], and an AO of this chemistry has now undergone Phase I clinical trials [101, 102].

There are advantages and disadvantages to each of the AO chemistries, and several factors must be taken into account. The LNA compound designed to excise exon 46 was able to induce substantial exon skipping, has the additional advantages of increased resistance to nuclease degradation and an exceptional affinity for the target sequence. A 15mer targeting exon 46 was estimated to have a Tm of 131°C [72], and it was the latter feature that raised concerns for potential off-target annealing, particularly after the authors showed that an AO with 2 mismatches was still able to induce targeted exon skipping [72]. We have shown that 2OMe AOs containing several mismatches could also induce targeted exon skipping *in vitro*, but this was only after application of high concentrations of AO, and skipping was not efficient compared to optimally designed AOs [85].

Although earlier studies reported that PNAs may be of limited use as agents to induce exon skipping [72, 100], this may again reflect limitations of delivery of the PNA into the nucleus, necessary for splice intervention. Recently, a report by Yin *et al* [103] indicated that PNAs of 20 bases long could induce substantial exon 23 skipping in both *mdx* cells *in vitro* and *in vivo* after intramuscular injection. These authors were able to compare efficiencies of different oligomer chemistries and concluded that the PMOs were marginally more efficient than the PNAs. It should be noted that direct sequence comparisons were not reported.

One limitation of the PMO chemistry is poor uptake *in vitro*, unless either very high concentrations were used or the cells were encouraged to take up the PMO by scrape loading [104]. Sense strand oligonucleotide leashes, designed to anneal to a PMO directed at mouse exon 23 donor, allowed the uncharged PMO to be complexed with a cationic liposome, and induced targeted exon removal at concentrations three orders of magnitude lower than the uncomplexed PMO in cell culture [100]. Once the PMOs were taken up by the cells, high levels of exon skipping were maintained for the life of the cultures, as these uncharged compounds are not metabolized. The PMO chemistry does not show any overt toxicity *in vitro*, even when added to cultures at concentrations of 50μM. More importantly, no serious drug-related adverse events have been observed in 15 safety studies of 4 different PMOs, designed as antiviral and metabolism modifying agents, involving approximately 350 individuals [105].

It is not realistic to consider using cationic liposome preparations for repeated systemic delivery, based upon cost and more importantly, potential toxicity. The pluronic block co-polymer F127 was shown to enhance uptake of 2OMe AOs [106, 107] and Wells *et al* [108] showed enhanced AO delivery in the mouse using electroporation. However, it is possible that systemic delivery may not be as great a challenge for PMOs as first anticipated. *In vivo* administration of a PMO was undertaken by injecting a cationic lipoplex composed of a PMO annealed to a sense strand leash, (1-5 ug) directed to the donor splice site of mouse dystrophin exon 23. As anticipated from *in vitro* studies, substantial dystrophin exon skipping was detected at the RNA and protein levels [100]. Immunofluorescence indicated that the induced dystrophin was correctly localized and the sarcolemmal complex was re-established. What was unexpected was that similar levels of exon skipping were induced after administration of an equivalent amount of PMO that was not annealed to the leash. It quickly became apparent that *in vivo* PMO uptake was much more efficient than anticipated from *in vitro* studies. Systemic studies followed and dystrophin expression could be detected in all tissues examined, except the heart [109, 110]. Further advances in PMO delivery came with conjugation to cell penetrating peptides, and even more substantial dystrophin expression was induced, using even lower doses of the PMO [111]. However, to date, the heart remains resistant to AO-induced exon skipping, prompting additional studies using different peptide tags and dosage regimens.

ALTERNATIVE EXON SKIPPING STRATEGIES

Several approaches are being investigated as potential avenues to induce permanent exon skipping. Gene editing using chimeric RNA/DNA oligonucleotides (RDOs) [112, 113], single stranded oligodeoxynucleotides (ODNs) [114], and plasmid DNA [115] has been reported. If a base change could be introduced at a donor or acceptor splice site, the modified cell could maintain permanent exon skipping. Although, the most common consequence of a splice site mutation is exon skipping, such as found in the canine model of muscular dystrophy [39], there are many instances where a donor or acceptor mutation has led to the activation of a cryptic splice site, thereby causing intron retention or partial exon loss in the mature mRNA. We previously reported a case of germline mosaicism in a family with a defect in the exon 26 donor splice site [116]. This mutation did not lead to loss of exon recognition and skipping, but rather activation of a cryptic splice site downstream, with intron retention and an in-frame stop codon now in the mature mRNA. It may be difficult to predict the consequences of each splice motif mutation until it has been induced and validated in human cells. What is clear is that levels of induced gene correction reported are generally very low, and vary extensively from one laboratory to another and this avenue of therapy is many years from the clinic [117, 118].

Viral vectors are being developed to introduce expression cassettes that allow synthesis of antisense RNA sequences [119, 120]. Auxiliary sequences such as U1 and U7 are proposed to enhance accessibility of the AO to the splice site. Goyenvalle *et al* [121] presented elegant work showing long-term dystrophin expression in the *mdx* mouse after introducing a viral construct carrying sequences annealing to the branch point of intron 22 and donor site of exon 23, linked to a modified U7 sequence under control of the U7 promoter. Dystrophin was readily detectable by western blotting and immunostaining 3 months after treatment. Should the appropriate construct be introduced into a stem or progenitor cell[122], proliferation capacity could allow for potentially enhanced therapeutic benefits.

Autologous cell therapy is also being investigated to restore dystrophin [123, 124]. Cells were harvested and then transfected with a lentiviral construct, containing an exon skipping cassette designed to constantly generate RNAs to dislodge the target exon. The treated autologous cells were evaluated for exon skipping and transplanted into the tibialis anterior of *mdx*/SCID mouse. A few dystrophin positive myofibres were detected 10 weeks after transplantion [123], and it appears that the low levels of dystrophin expression was caused by a combination of low exon skipping efficiency and poor viability of transplanted cells.

CLINICAL TRIALS

The first clinical trial to address a DMD-causing mutation by induced exon skipping involved a single patient with a frame-

shifting deletion of exon 20. An oligomer, directed to exon 19, should restore the reading frame in this individual [94]. The treatment consisted of an ODN administered intravenously at a dosage of 0.5 mg/kg of body weight per week, for 4 weeks. Dystrophin protein was reportedly detected at very low levels by immunostaining of sections from the patient's biceps, 1 week after the last infusion. No western blot data was shown and the treatment failed to reduce serum creatine kinase levels. Although exon skipping was demonstrated in lymphocytes after the third and fourth treatments, only low levels of transcript missing exons 19 and 20 were found in the muscle biopsy after 4 treatments. In this report, preclinical testing was limited to one species, the *mdx* mouse, in which doses of 200mg/kg were administered by infusion. There were no adverse effects reported, but surprisingly neither were any exon skipping studies, since this particular oligomer matched the mouse dystrophin sequence perfectly and had been shown to induce mouse exon skipping *in vitro* [85]. Even more surprising, this compound had previously been reported to induce exon 19 skipping in the *mdx* mouse, but only after intraperitoneal injection [125]. Although a similar infusion protocol was used to administer this compound to a normal human volunteer for safety testing, no exon skipping analysis was reported.

This raises one of the fundamental problems with pre-clinical testing oligomers designed for dystrophin exon skipping. If this compound had efficiently dislodged exon 19 from the dystrophin pre-mRNA of the normal human volunteer, the reading frame will be disrupted, leading to reduced dystrophin expression, and in essence induce muscular dystrophy. This aspect is more discussed in detail below.

In addition, highly sensitive assays could lead to misinterpretation of exon skipping efficiency. Lymphocytes have been used to study illegitimate dystrophin expression [126], where it has been estimated that one copy of dystrophin gene transcript occurs in about one thousand cells [127]. As very sensitive assays were required to detect these illegitimate transcripts, the low level of exon 19 skipping from the muscle biopsy is unlikely to reflect an accurate ratio of rescued muscle gene transcript. Several issues relating to dosage and route of administration, which were not properly addressed, highlight some limitations of this clinical trial.

A Phase I clinical trial in Leiden has now been completed [102]. A 2OMe AO designed to induce exon 51 skipping was injected into tibialis anterior muscles of 4 DMD patients. Exon 51 was chosen as the target for this study as its removal would correct the reading frame in more DMD individuals than any other exon, according to the Leiden muscular dystrophy database (http://www.dmd.nl/). Preexisting or fibroblast derived myogenic cells from patients were used for *in vitro* pre-screening of oligomer PRO051 [102]. Four weeks after four intramuscular injections of 200 μg of PRO051 oligomer, running along a 1.5cm measuring line, muscle from the 8 to 12 year old participants were assessed for exon skipping at the RNA level and dystrophin restoration. Substantial dystrophin restoration was demonstrated in all patients by both immunostaining and western blot analysis [102]. This is the first evidence to conclusively support the potential of using AOs to restore dystrophin in DMD patients. Another trial has recently commenced injecting patients in the United Kingdom, also with the aim of inducing exon 51 excision. However, there are several differences from the above study, including oligomer sequence [89], oligomer chemistry and dosage, and the muscle to be treated [101].

FUTURE CHALLENGES

There is considerable optimism that AO-induced exon skipping may substantially reduce the progression and symptoms of DMD. However, a number of major challenges lie ahead. First, the classification of exon skipping as a gene therapy is regarded differently by regulatory agencies in different countries. We propose that the use of AOs to induce exon skipping should not be regarded as a form of gene therapy, since no permanent genetic changes are induced in the recipient. These oligomers cannot integrate into the DNA, and their mode of action interferes with gene expression, not the gene. In some respects, oligomers inducing exon skipping should be viewed no differently to a compound such as PTC124, which can suppress premature termination codons [10], or any other molecule or antibody that blocks or modifies the function of a gene product. Upon cessation of the AO administration, the compounds will be either degraded by endogenous nucleases or gradually cleared from the system. No permanent changes will have been introduced, and while this may expedite some regulatory aspects of the work, it also poses a potential limitation, as the AOs will need to be re-administered at periodic intervals to maintain therapeutic levels of the induced protein.

The initial exon 51 skipping trials in Leiden and the United Kingdom will only provide proof-of-principle, with relatively limited information on safety being generated, since low doses of AOs of two chemistries are administered by an intramuscular injection. This mode of delivery cannot be considered to treat the entire body, although it may be possible to treat individual muscles in the hand, wrist, and forearm of older boys. This may preserve what little muscle is left and enhance their function.

The extent of the genomic deletion causing DMD will significantly influence the functionality of the AO-induced dystrophin isoform. The dystrophin isoform rescued by skipping of exon 51 in a DMD individual carrying a genomic deletion of exon 50 is likely to be more functional than the isoform induced in a patient whose gene lesion extended from exon 13 to 50. Targeted excision of exon 51 would restore the reading frame in both deletions, but the missing coding region from the larger deletion would result in a greatly shortened dystrophin isoform. Genomic deletions in excess of 36 exons have generally been associated with a severe phenotype, regardless of the reading frame [128]. It is to be expected that different BMD-like dystrophin isoforms will have variable function, which would in turn influence the stability and rate of turn-over of the protein in muscle and presumably, the muscle fibres.

While different DMD patients with the same type of genomic mutation could be treated with the same AO preparation, the different dystrophic individuals may require oligomer dosage regimens that will be determined by their genetic background and nature of the dystrophin mutation. The influence of genetic backgrounds on manifestation of the same dystrophin mutation within one family has been reported [50]. Prescreening the target exons in recipients will then be essential to ensure there are no neutral DNA polymorphisms that could compromise AO annealing and hence excision of the targeted exon.

The cost of bringing a single drug to the market can be hundreds of millions of dollars [129]. In the case of drugs to treat common conditions, it would be expected that these may be used by millions of people. In these situations, extensive testing is mandatory to identify any adverse effects, particularly considering the number of individuals being exposed. The potential cost of bringing 12 different AOs, which would treat the majority of DMD deletion patients, to the market is staggering and will be beyond the capacity of any organizations other than the largest pharmaceutical companies. It should be noted that the 'majority of deletion patients' would still only constitute about 60% of all DMD individuals, and a proportion of these would carry such large deletions, or the loss of crucial coding domains, such that exon skipping may not be a viable option.

The non-deletion DMD individuals will require many different AOs to address their mutations, as such lesions are scattered across the dystrophin gene. The concept of developing over 100 antisense compounds seems ludicrous, yet this must be considered if exon skipping is to be applicable to all amenable dystrophin mutations,

especially since many of these defects occur in the large central rod domain. The loss of a single exon in the rod domain is expected to result in a dystrophin isoform of near normal function, since this is a variably dispensable part of the dystrophin protein. If AOs are to be used as a personalized genetic medicine, some compounds may be designed to treat a mutation found in only a single family. We have shown that targeting dystrophin pseudo-exons with AOs can block their inclusion in the mature mRNA [54, 130]. Unlike restoring the reading frame around a genomic deletion or excising exons carrying nonsense mutations, AO-induced pseudo-exon suppression could lead to the production of a perfectly normal dystrophin. If the priority of targeted exon skipping is only based upon the frequency of mutations, then pseudo-exons would never be considered, despite being potentially the most responsive type of dystrophin gene defect.

Several PMOs of different sequences have been tested in animals, including mouse, rat, dog, and non-human primate for general toxicity and side-effects that may be associated with the backbone chemistry [105]. While safety studies of oligomers designed for viral gene suppression have been undertaken in normal human volunteers, similar safety trials cannot be considered for testing AVI-4658, a PMO designed to excise dystrophin exon 51. Evaluating AVI-4658 in normal human volunteers must be regarded as unethical due to unacceptable risks to the participants. If this compound works exactly as predicted, exon 51 would be removed from the normal dystrophin gene transcript, disrupting the reading frame and potentially inducing DMD. As shown in Fig. (2), the consequences of exon 51 skipping will vary extensively between normal individuals and different DMD patients. In what should be a most amenable mutation (DMD Δ50), AVI-4658 would restore the reading frame around this single exon deletion, and allow synthesis of a dystrophin of near-normal length and function. The nature of induced dystrophin isoform will depend upon the extent of the primary gene deletion, hence potential benefits of exon skipping would be compromised by larger gene deletions; such as DMD Δ30-50 and Δ13-50. Thus, there is a broad spectrum of potential consequences of administering AVI-4658 to different individuals, ranging from no restoration of the reading frame in DMD patients with non-responsive mutations, induction of dystrophin isoforms of variable function depending upon the primary gene lesion, to inducing DMD in a normal individual by disrupting the dystrophin reading frame.

Although non-human primate studies may be more relevant, there are limitations, as there are no known primate models with dystrophin genomic deletions that would be restored by exon 51 skipping. Similarly, removal of a dystrophin exon could disrupt the reading frame and induce an adverse reaction associated with dystrophin deficiency, since the exon skipping compound would be working exactly as it was designed to do. Animal testing of compounds designed for specific human dystrophin mutations can only supply limited information, and should be only undertaken if relevant data is generated.

There is currently no therapy available for DMD that addresses the missing or defective dystrophin. Although corticosteroids such as prednisolone or deflazacort have been shown a clear benefit in slowing muscle wasting [131-133], mood swings, weight gain, stunted growth, brittle bone and cataracts have inevitably become acceptable side effects of the treatment. It is imperative that as many therapeutic compounds are made available to the DMD community in shortest possible time frame. DMD is a relentless progressive muscle wasting disorder that does not wait for regulatory approval, challenges in oligomer design, delivery, and production.

For the widespread implementation of oligomer induced splice intervention as a therapy for DMD, it may become necessary to regard induced exon skipping as a generic platform. If the first

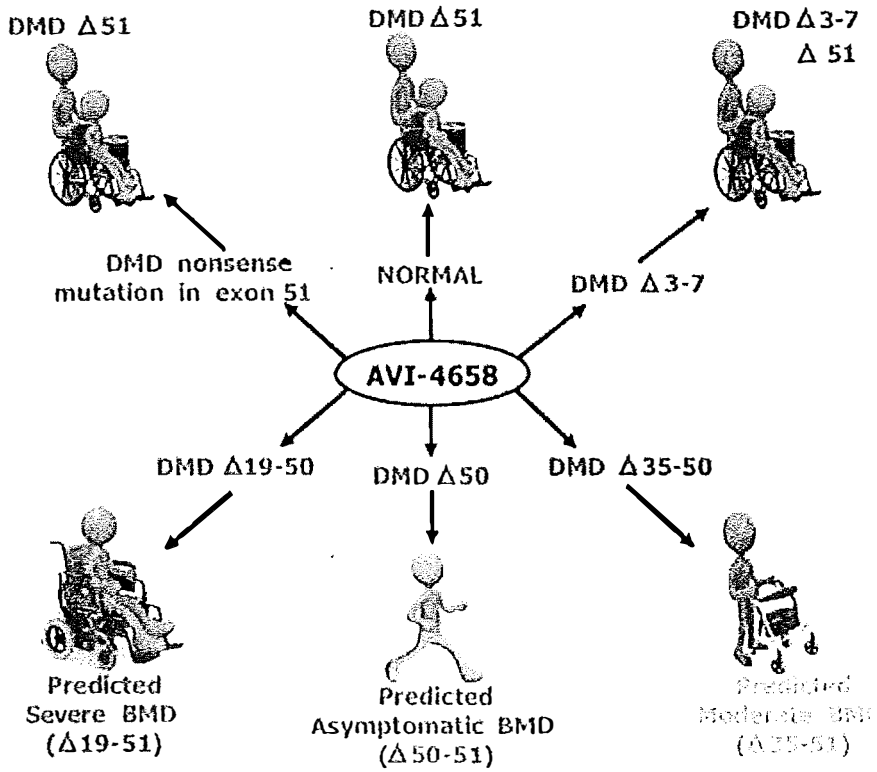

Fig. (2). Potential consequences of AO induced exon 51 skipping in individual with different dystrophin gene. Δn indicates deleted exons before and after treatment. Δ19-51, Δ35-51, and Δ50-51 are all in-frame transcripts and should lead to various BMD phenotype.

clinical trials show safety and efficacy in restoration of dystrophin expression after excising exon 51 from some patients, there must be a move to systemic administration and developing therapeutic dosage regimens. This is likely to take some considerable time, and may be confounded by the nature of the primary gene lesion and the genetic background of the patient. At the same time, additional exon targets must be considered to address other DMD mutations, and in this manner, sufficient safety data would become available that could allow different oligomer sequences of a particular class, for example PMOs, to be regarded as class-specific compounds.

Steroids, the current "gold standard" treatment to delay DMD progression, exert their effect through an unknown mechanism and have been available for decades. Despite this, there is still no consensus on the best dose and treatment regimen for steroids. Faced with the challenge of developing a personalized genetic intervention to address many different dystrophin mutations, it is most likely that establishing oligomer dosage regimens for individual DMD patients will prove to be an even greater challenge. Nevertheless, upon the demonstration of one mutation being amenable to exon skipping, we must make all efforts to expedite the application to as many different dystrophin mutations as possible.

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ABBREVIATIONS

AO	=	antisense oligomer
BMD	=	Becker muscular dystrophy
cDNA	=	complementary DNA
DMD	=	Duchenne muscular dystrophy
DNA	=	deoxyribonucleic acid
ENAs	=	4'-C-ethylene bridge nucleic acids
ESE	=	exon splicing enhancer
GRMD	=	Golden retriever muscular dystrophy
kDa	=	kilodalton
LNAs	=	locked nucleic acids
MOE	=	2'-O-methoxy-ethoxy AO
NMD	=	nonsense mediated decay
ODN	=	oligodeoxynucleotide
PMO	=	Phosphorodiamidate Morpholino Oligomer
PNAs	=	peptide nucleic acids
pre-mRNA	=	precursor messenger ribonucleic acid
RNA	=	ribonucleic acid
SCID	=	severe combined immunodeficiency
2OMe	=	2'-O-methyl modified

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RESEARCH ARTICLE

By-passing the nonsense mutation in the 4^{CV} mouse model of muscular dystrophy by induced exon skipping

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Abstract

Background Duchenne muscular dystrophy (DMD), a severe neuromuscular disorder, is caused by protein-truncating mutations in the dystrophin gene. Absence of functional dystrophin renders muscle fibres more vulnerable to damage and necrosis. We report antisense oligomer (AO) induced exon skipping in the B6Ros.Cg-Dmd$^{mdx-4Cv}$/J (4^{CV}) mouse, a muscular dystrophy model arising from a nonsense mutation in dystrophin exon 53. Both exons 52 and 53 must be excised to remove the mutation and maintain the reading frame.

Methods A series of 2′-*O*-methyl modified oligomers on a phosphorothioate backbone (2OMeAOs) were designed and evaluated for the removal of each exon, and the most effective compounds were then combined to induce dual exon skipping in both myoblast cultures and *in vivo*. Exon skipping efficiency of 2OMeAOs and phosphorodiamidate morpholino oligomers (PMOs) was evaluated both *in vitro* and *in vivo* at the RNA and protein levels.

Results Compared to the original *mdx* mouse studies, induction of exon skipping from the 4^{CV} dystrophin mRNA was far more challenging. PMO cocktails could restore synthesis of near-full length dystrophin protein in cultured 4^{CV} myogenic cells and *in vivo*, after a single intramuscular injection.

Conclusions By-passing the protein-truncating mutation in the 4^{CV} mouse model of muscular dystrophy could not be achieved with single oligomers targeting both exons and was only achieved after the application of AO cocktails to remove exons 52 and 53. As in previous studies, the stability and efficiency of PMOs proved superior to 2OMeAOs for consistent and sustained protein induction *in vivo*.

Keywords antisense oligomer; B6Ros.Cg-Dmd$^{mdx-4Cv}$/J (4^{CV}) mouse; Duchenne muscular dystrophy; exon skipping; morpholino

Introduction

Duchenne muscular dystrophy (DMD), a devastating neuromuscular disorder characterized by progressive muscle wasting, and proximal muscle weakness, arises from the absence of functional dystrophin. Dystrophin links the actin cytoskeleton to the extracellular matrix via a complex of proteins embedded in the sarcolemma and plays a pivotal role during muscle contraction [1–3]. Loss of dystrophin renders muscle fibres vulnerable to membrane damage

during contraction. Progressive loss of muscle fibres, with inflammatory cell infiltration and fibrosis eventually overwhelms the regenerative capacity of the muscle.

The human dystrophin gene is the largest known, and the major muscle isoform consists of 79 exons, spanning 2.4 million bp. Most mutations in DMD patients are intragenic deletions or duplications, accounting for approximately 60% and 8% of all DMD patients, respectively [4–7]. Point mutations, including nonsense and splice motif mutations, as well as small insertions/deletions that disrupt the reading frame, are responsible for 25–35% of all cases [7,8]. DMD mutations typically disrupt the reading frame, thereby preventing synthesis of functional dystrophin. Becker muscular dystrophy (BMD) is also caused by mutations in the dystrophin gene but this milder allelic condition is generally caused by gene defects that do not disrupt the reading frame and allow production of shorter, but partially functional, protein. Depending upon the position and nature of the mutation, some cases of BMD may only be diagnosed late in life, and present with very mild or no symptoms [9,10], whereas others may present as borderline DMD and lose ambulation around the age of 15 years [11,12].

AO-induced exon skipping studies initially targeted different splice motifs of exon 23 in the muscular dystrophy mouse model (*mdx*), with the aim of restoring protein expression [13,14]. The defect in the *mdx* mouse is a naturally occurring nonsense mutation in dystrophin exon 23. Despite limitations, including a mild clinical phenotype, the *mdx* mouse has been widely used in developing potential therapies for DMD, including exon skipping [15–22], gene and cell replacement [23,24], and premature translation termination suppression [25].

In the present study, the B6Ros.Cg-Dmd$^{mdx-4Cv}$/J (4^{CV}) muscular dystrophy mouse [26], carrying a nonsense mutation in exon 53 of the dystrophin gene, was used to evaluate AO-induced dual exon skipping in a region of the dystrophin gene within the major human dystrophin deletion hot-spot. By-passing the 4^{CV} mutation, and maintaining the reading frame, requires removal of both exons 52 and 53 from the mature dystrophin gene transcript. A series of AOs were designed and evaluated for the removal of each exon, and the most effective compounds were then combined to induce dual exon skipping in both myoblast cultures and *in vivo*. AOs of two different chemistries, 2′-*O*-methyl modified oligomers on a phosphorothioate backbone (2OMeAOs), and phosphorodiamidate morpholino oligomers (PMOs) conjugated to a cell-penetrating peptide (P007) [27,28] were compared.

Materials and methods

AOs and primers

AO nomenclature is based on that described by Mann *et al.* [18]. Sequences and composition of AO treatments are described in Table 1. 2OMeAOs were synthesized on an Expedite 8909 Nucleic Acid synthesizer (Applied Biosystems, Foster City, CA, USA) using the 1 μmol thioate synthesis protocol. AOs were designed to anneal to either exonic sequences or exon/intron junctions of mouse dystrophin exons 52 or 53. PMOs conjugated to an arginine-rich, cell penetrating peptide (P007) [27,28] were synthesized by AVI BioPharma (Corvallis, OR, USA). Primers for RT-PCR and sequencing analysis were synthesized by Geneworks (Adelaide, Australia) and are listed in the Supporting information (Table S1).

Animals

4^{CV} (B6Ros.Cg-Dmd$^{mdx-4Cv}$/J) congenic mice, obtained from the Jackson Laboratory (Bar Harbor, ME, USA), were raised and supplied by the Animal Resources Centre, Murdoch, Western Australia. Animal housing and transport followed guidelines from National Health and Medical Research Council (Australia). The use of animals was approved by the Animal Ethics Committee of University of Western Australia (approval number 03/100/572).

Cell culture and AO transfection

Immortalized *mdx H2K^b-tsA58* mouse cells (*H-2K mdx*) were propagated and transfected as described previously [29]. Primary myoblast cultures were prepared from 2–4-day-old 4^{CV} pups and the procedure was adapted from Rando *et al.* [30]. Limb muscles from four pups were dissected, homogenized, and incubated at 37°C for 30 min with dissociating enzyme mix containing 2.4 units/ml dispase (Invitrogen, Victoria, Australia), 5 mg/ml collagenase Type II (Invitrogen), and 2.4 mM $CaCl_2$ in Dulbecco's modified Eagle's medium (Invitrogen). After centrifugation, the cell pellet was added to a 75-cm^2 tissue culture flask with 10 ml of proliferative media and incubated for 1 h. Non-adherent cells were removed and seeded into 75-cm^2 tissue culture flasks coated with 100 μg/ml matrigel. When nearly confluent, cells were seeded into 24-well plates coated with 50 μg/ml poly D-lysine solution and matrigel, and incubated for 48 h before transfection. Duplicate wells were transfected with 2OMeAO lipoplexes using Lipofectin (Invitrogen) at a ratio of 2 : 1 Lipofectin to AO. Briefly, Lipofectin was mixed with OptiMEM (Invitrogen) to a final volume of 200 μl and incubated for 30 min at room temperature. The 2OMeAO, which had been diluted to 200 μl in OptiMEM, was then combined with Lipofectin : OptiMEM and the mixture incubated for a further 30 min, before addition of OptiMEM to a final volume of 1 ml and subsequent addition of 500 μl aliquots to each well. Transfected cells were incubated for 48 h before RNA was extracted for analysis.

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Intramuscular administration

Oligomers, in physiological saline, were injected into tibialis anterior (TA) muscles at doses indicated. Each experiment included at least one saline only injection as a negative control. The animals were anaesthetized and sacrificed by cervical dislocation at indicated time points after the injection, and muscles were removed and snap-frozen in pre-cooled isopentane, before being sectioned and prepared for RNA and protein studies.

RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, and DNA sequencing

RNA was harvested from *H-2K mdx* cultures, 4^{CV} cultures and frozen sections tissue blocks using Trizol (Invitrogen), according to the manufacturer's protocol. One-step RT-PCR was undertaken using 120 ng of total RNA as template, in a 12.5 µl reaction for 30 cycles of amplification. After the reverse transcription step for 30 min at 55 °C, the reaction was heated to 94 °C for 2 min before the primary thermal cycling rounds of 94 °C for 40 s, 60 °C for 1 min, and 68 °C for 1 min. Nested PCR was then carried out on 1 µl of the primary amplification reaction using AmpliTaQ Gold (Applied Biosystems, Foster City, CA, USA). Cycling conditions for the secondary PCR were 94 °C for 6 min to activate the polymerase, followed by 20 cycles of 94 °C for 40 s, 60 °C for 1 min, and 72 °C for 1 min. PCR products were separated on 2% agarose gels in TAE buffer and the images captured on a CHEMISMART-3000 (Vilber Lourmat, Marne-La-Vallee, France) gel documentation system. Bands of interest were re-amplified directly from the agarose gel [31] and the sequencing templates were purified using UltraClean spin columns (Mobio Laboratories, Carlsbad, CA, USA) and then sequenced on an ABI 377 automated sequencer using BigDye v3.1 terminator chemistry (Applied Biosystems).

Western blot analysis

Protein extracts were prepared as weight per volume of treatment buffer containing 125 mM Tris/HCl, pH 6.8, 15% sodium dodecyl sulphate, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM dithiothreitol, bromphenol blue, and protease inhibitor cocktail (Sigma, St Louis, MO, USA) to cell pellet or mouse muscle cryostat sections. Samples were then vortexed and sonicated briefly, heated at 95 °C for 5 min, before electophoretic fractionation on a 4–10% sodium dodecyl sulphate gradient gel at pH 8.8 with 4% stacking gel, pH 6.8. Densitometry of myosin bands after coomassie blue staining was undertaken to facilitate equivalent protein loading. Extracts from AO treated cultures or muscle cryosections from treated animals (2.75 mg) and control muscle (0.275 mg) were loaded onto a second polyacrylamide gel electrophoresis gel for western blotting. Proteins were transferred from the gel to nitrocellulose membranes (Amersham Biosciences, Castle Hill, Australia) overnight at 18 °C, at 290 mA. Dystrophin was visualised using NCL-DYS2 monoclonal anti-dystrophin antibody (Novocastra, Newcastle-upon-Tyne, UK) as described previously [21]. Images were captured on a Vilber Lourmat CHEMISMART-3000 gel documentation system. The percentage of dystrophin restoration was calculated according to dystrophin expression in control cells after normalization for myosin loading.

Tissue preparation and immunofluorescence

TA muscles were taken from mice and snap-frozen in isopentane, pre-cooled in liquid nitrogen. Dystrophin was detected in 6 µm unfixed cryostat sections using NCL-DYS2, an antibody that reacts strongly with C-terminus of dystrophin. Immunofluorescence was performed using the Zenon Alexa Fluor 488 labelling kit (Invitrogen), according to the manufacturer's protocol, except for the initial fixation step. The primary antibody was diluted at 1 : 10 and sections were counterstained with Hoechst (Sigma) at the dilution of 1 : 10 000 to visualize nuclei. Sections were viewed under Olympus IX 70 inverted microscope (Olympus, Tokyo, Japan) and images were captured on an Olympus DP70 digital camera.

Results

Single exon targeting

A panel of AOs was designed to anneal to obvious splicing motifs, including the acceptor and donor splice sites, and potential exonic splicing enhancers (ESEs) of both exons 52 and 53. Although designed to predict human ESEs, the web-based ESE prediction program, ESEfinder 3.0 [32,33], was used to identify putative ESEs in mouse dystrophin exons 52 and 53. Figure 1 shows the predicted ESEs of exons 52 and 53 and the relative annealing positions of the AOs (sequences are provided in Table 1) designed to induce exon removal. Six AOs, designed to excise dystrophin exon 52, were evaluated initially in *H-2K^{b}-tsA58 mdx* (*H-2K mdx*) immortalised myogenic cells [34]. The use of these immortalized cells reduced animal usage and, because the 4^{CV} mutation in exon 53 did not alter dystrophin splicing patterns, it was assumed that design in one mouse strain should be valid for another. Subsequent experiments validated this approach. According to ESEfinder, the nonsense mutation in dystrophin exon 53 occurs in two predicted ESE motifs that are only just above the threshold: an SF2/ASF motif (score = 2.187/threshold = 1.956) and an SRp40 motif (score = 3.042/threshold = 2.67).

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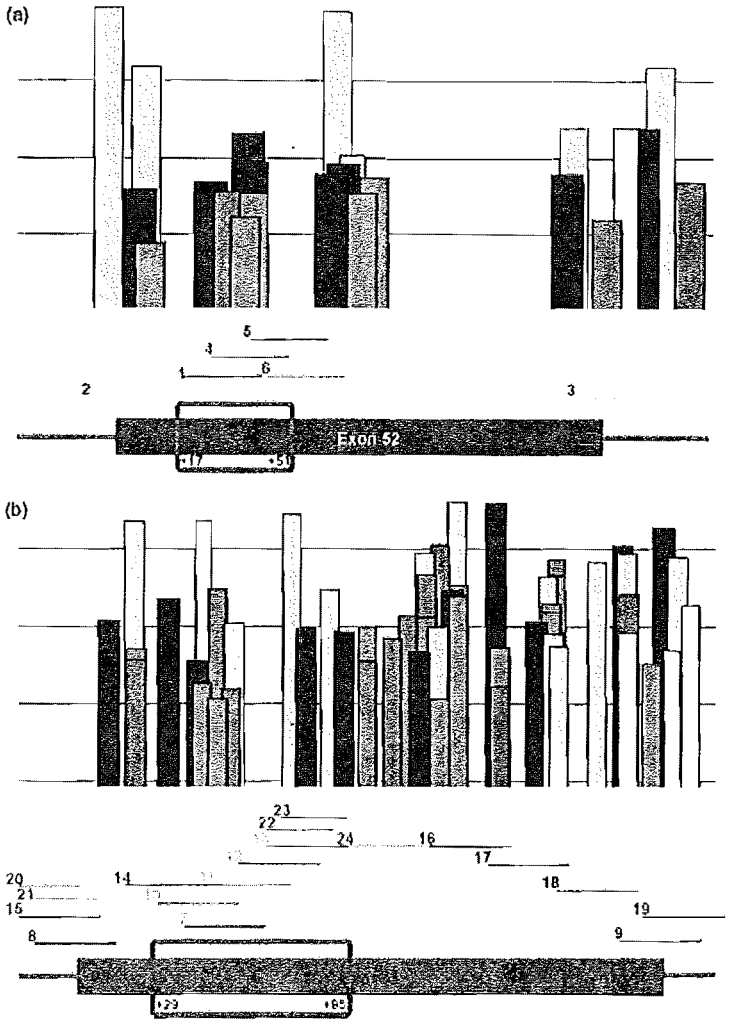

Figure 1. Diagrammatic presentation of predicted ESEs [32,33]; (a) mouse exons 52 and (b) exon 53. Relative annealing coordinates of oligomers are indicated

Table 1. Sequences, annealing coordinates, length and GC content of all AOs targeting exons 52 and 53

AO	Length (bp)	Annealing coordinates	Exon number	Sequences	GC content (bp)	%
1	25	M52A + 17 + 41	52	5′-UCC AAU UGG GGG CGU CUC UGU UCC A-3′	14	56
2	30	M52A − 15 + 15	52	5′-AUC UUG CAG UGU UGC CUG AAA GAA AAA AAA-3′	10	33
3	30	M52D + 15 − 15	52	5′-CAU UAA GAG ACU UAC UUC GAU CAG UAA UGA-3′	10	33
4	30	M52A + 22 + 51	52	5′-AAU GAG UUC UUC CAA UUG GGG GCG UCU CUG-3′	15	50
5	30	M52A + 32 + 61	52	5′-GGG CAG CAG UAA UGA GUU CUU CCA AUU GGG-3′	15	50
6	30	M52A + 42 + 71	52	5′-UUC AAA UUC UGG GCA GCA GUA AUG AGU UCU-3′	12	40
7	31	M53A + 39 + 69	53	5′-CAU UCA ACU GUU GUC UCC UGU UCU GCA GCU G-3′	15	48
8	30	M53A − 15 + 15	53	5′-UCU GAA UUC UUU CAA CUG GAA UAA AAA UAA-3′	7	23
9	30	M53D + 15 − 15	53	5′-AUG CUU GAC ACU AAC CUU GGU UUC UGU GAU-3′	12	40
10	30	M53A + 29 + 58	53	5′-UGU CUC CUG UUC UGC AGC UGU UCU UGA ACC-3′	15	50
11	30	M53A + 49 + 78	53	5′-UUA ACA UUU CAU UCA ACU GUU GUC UCC UGU-3′	10	33
12	30	M53A + 59 + 88	53	5′-GUU GAA UCC UUU AAC AUU UCA UUC AAC UGU-3′	9	30
13	30	M53A + 69 + 98	53	5′-CAG CCA UUG UGU UGA AUC CUU UAA CAU UUC-3′	11	37
14	30	M53A + 19 + 48	53	5′-UCU GCA GCU GUU CUU GAA CCU CAU CCC ACU-3′	15	50
15	30	M53A − 25 + 5	53	5′-UUC AAC UGG AAU AAA AAU AAG AAU AAA GAA-3′	6	20
16	30	M53A + 129 + 158	53	5′-CCA UGA GUC AAG CUU GCC UCU GAC CUG UCC-3′	17	57
17	30	M53A + 151 + 180	53	5′-CUA CUG UGU GAG GAC CUU CUU UCC AUG AGU-3′	14	47
18	30	M53A + 176 + 205	53	5′-UCU GUG AUC UUC UUU UGG AUU GCA UCU ACU-3′	11	37
19	30	M53D + 5 − 25	53	5′-UUU UAA AGA UAU GCU UGA CAC UAA CCU UGG-3′	10	33
20	25	M53A − 25 − 1	53	5′-CUG GAA UAA AAA UAA GAA UAA AGA A-3′	5	20
21	25	M53A − 20 + 5	53	5′-UUC AAC UGG AAU AAA AAU AAG AAU A-3′	5	20
22	25	M53A + 69 + 93	53	5′-AUU GUG UUG AAU CCU UUA ACA UUU C-3′	7	28
23	25	M53A + 74 + 98	53	5′-CAG CCA UUG UGU UGA AUC CUU UAA C-3′	10	40
24	25	M53A + 99 + 123	53	5′-CCU GUU CGG CUU CUU CCU UAG CUU C-3′	13	52

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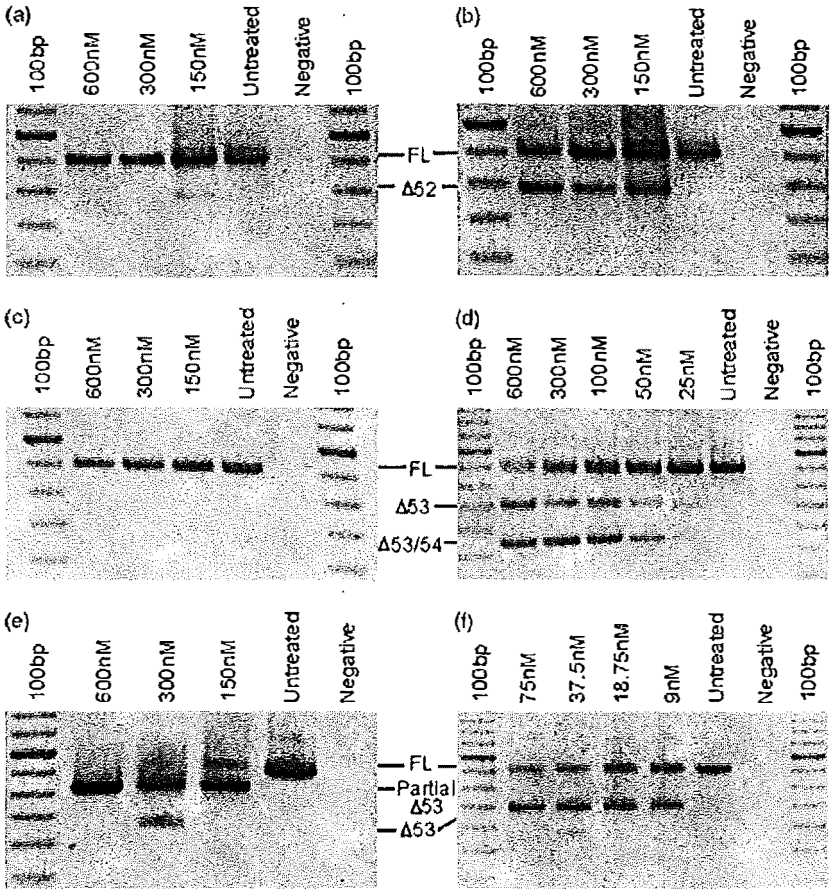

Figure 2. Induced single exon skipping in *H-2K mdx* cells; (a) Transcripts resulting after transfection with AO 2 (typical of that obtained after transfection with AOs 3, 5 and 6); (b) Transcripts resulting after transfection with AO 4 and AO 1; (c) Transcripts resulting after transfection with AO 8 (typical of that obtained after transfection with AOs 9, 14, 15, 16 and 19–24); (d) Transcripts resulting after transfection with AO 7 (typical of that obtained after transfection with AOs 7 and 10–13); (e) Transcripts resulting after transfection with AO 17; (f) Transcripts resulting after transfection with cocktail AOs 8, 9 and 13

None of the AOs targeting the acceptor or donor sites of exon 52 consistently induced removal of the target (Figure 2a), whereas the AOs annealing between the coordinates +17 + 51 efficiently excluded exon 52 from the mature dystrophin mRNA (Figure 2b). Subsequent titration studies comparing AOs 1 and 4 indicated that the latter compound was more efficient at inducing exon 52 removal at lower transfection concentrations, although there was little exon 52 skipping induced at 25 nM (data not shown). Consequently, AO 4 was selected for further studies.

Excision of exon 53 proved to be a greater challenge, and although numerous AOs were designed and evaluated, no single AO tested efficiently excluded exon 53 from the mouse dystrophin mRNA. Although many AOs failed to dislodge exon 53, as shown in Figure 2c, others removed exon 53 in addition to both exons 53 and 54 (Figure 2d). Two AOs activated a cryptic splice site and resulted in partial exon 53 loss (Figure 2e). Thirteen different combinations of non-overlapping AOs were then assessed and one AO cocktail was developed to consistently induce specific exon 53 skipping (Figure 2f).

Exon 52 and 53 skipping: 2OMeAOs

As the relative efficiencies of excision of the target exons differed, it was necessary to evaluate combinations of the optimized AOs directed to exons 52 and 53 (Table 2). Four 2OMeAOs (4, 8, 9 and 13) were combined at ratios indicated (A1, A2, A3, A4 and A5) and used to transfect 4^{CV} cultured primary myoblasts (Figures 3a to 3c). The identity of the transcript missing exons 52 and 53 was confirmed by DNA sequencing (Figure 3d). At 2 days after transfection, the A2 and A3 cocktails appeared marginally more effective at excising both exons 52 and 53, as shown by the absence of the full-length product (Figures 3a to 3c); hence, the A2 cocktail was selected for further studies.

Although RT-PCR studies indicated substantial exons 52 and 53 skipping at days 3 and 5, only a trace of shortened transcript could be detected at days 8 and 9 after treatment (data not shown). No detectable dystrophin protein was observed in treated cultures at any time point. RNA and protein were analysed at day 14 after two transfections at days 0 and 9, and no exon skipping

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studies, restoration of dystrophin protein in 2OMeAO treated cultures was more problematic [35]. AOs 4, 8, 9 and 13 were prepared as PMOs, coupled to the cell penetrating peptide P007. These oligomers did not induce any exon skipping when applied individually (data not shown). The combination was evaluated in 4^{CV} myoblast cultures and, as with the 2OMe AOs, different ratios of PMOs were assessed. The PMO cocktails B2, B3 and B4 induced the transcript missing both target exons at concentrations as low as 0.5 μM *in vitro*. The B2 and B3 PMO combinations comparable to the A2 and A3 2OMe combinations appeared to be the most effective mixtures, and were selected for further study (Figures 3e to 3g). Both the B2 and B3 cocktails were able to induce pronounced exon 52 and 53 skipping, 2 weeks after *in vitro* transfection at a concentration of 40 μM (Figure 3h), consistent with the appearance of induced dystrophin, as determined by western blotting (Figure 3i). Normalization of dystrophin according to myosin densitrometry indicates the B2 and B3 cocktails induced 11% and 8% of normal levels of dystrophin, respectively (Figure 3i; see also Supporting information, Table S2).

Evaluation off-target effects

To confirm specificity of the multi-oligo cocktail on dystrophin expression, RT-PCR was undertaken across the dystrophin gene transcript using five sets of nested primer pairs (see Supporting information) covering exons 13–70 region (Figures 4a to 4e). Figures 4a to 4e represent amplified segments of the dystrophin transcript. Although sporadic 'revertant' transcripts were detected in PMO cocktail treated cultures, the numbers of alternatively spliced transcripts were not greatly different from those in six untreated cultures (Figure 5).

In vivo studies

A single injection of 40 μg of each of the PMO cocktails, at ratios shown in Table 2, was made into the TAs of 4^{CV} mice. Two weeks after injection, RT-PCR on RNA extracted from injected 4^{CV} muscle demonstrated exon 52 and 53 skipping in all cocktail-treated samples (Figure 6a) with western blotting indicating dystrophin expression to be approximately 5–7%, based upon normalization of loading (see Supporting information, Table S2). Consistent with the RNA studies, dystrophin immunofluorescence on sections from the *TAs* of 4^{CV} mice treated with PMO cocktails C1 and C2 also showed dystrophin-positive fibres (Figures 7a and 7b).

Discussion

Oligomer design, evaluation of different chemistries and systemic delivery protocols have been extensively studied in the *mdx* mouse model of muscular dystrophy [14,18–21,36,37]. The present study describes the application of AO-induced dual exon skipping to address the primary gene lesion in another mouse model, the 4^{CV} mouse, whose nonsense mutation should be by-passed with the excision of exons 52 and 53. This model may be regarded as of greater relevance to the human condition because the targeted exon skipping is induced within the major hotspot for dystrophin gene deletions [38].

Acceptor and donor splice sites were initially considered obvious targets for AO-induced exon skipping, and consistent exon 23 removal was achieved with the first

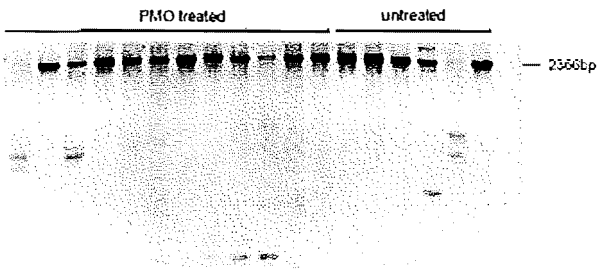

Figure 5. RT-PCR analysis of dystrophin transcripts in untreated and PMO treated 4^{CV} cultures across exons 20–35 indicating alternatively spliced dystrophin transcripts

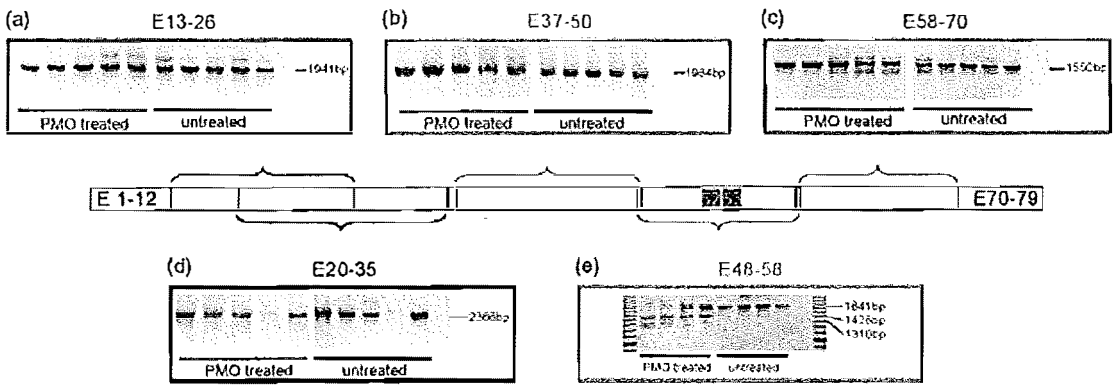

Figure 4. RT-PCR analysis of dystrophin transcripts in untreated and PMO treated 4^{CV} cultures across (a) exons 13–26, (b) exons 37–50, (c) exons 58–70, (d) exons 20–35 and (e) exons 48–58

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Table 2. Composition of AO cocktail: *in vitro* studies (2OMeAO and PMO) and *in vivo* studies (PMO)

Cocktail	A1	A2	A3	A4	A5
2OMe AO	1:3	1.66:1	3.33:1	6.66:1	12.33:1
4	150	375	460	520	555
8	150	75	46	26	15
9	150	75	46	26	15
13	150	75	46	26	15
Total concentration (mM)	600	600	600	600	600
Cocktail	B1	B2	B3	B4	B5
P007-PMO	1:3	1.66:1	3.33:1	6.66:1	12.33:1
4	2.5	6.25	7.7	8.8	9.25
8	2.5	1.25	0.77	0.44	0.25
9	2.5	1.25	0.77	0.44	0.25
13	2.5	1.25	0.77	0.44	0.25
Total concentration (μM)	10	10	10	10	10
Cocktail	C1	C2	C3	C4	C5
P007-PMO	1:3	1.66:1	3.33:1	6.66:1	12.33:1
4	10	25	30	35	37
8	10	5	3.3	1.8	1
9	10	5	3.3	1.8	1
13	10	5	3.3	1.8	1
Total amount (μg)	40	40	40	40	40

or restored dystrophin protein were observed (data not shown).

A total of 100 μg of the 2OMeAO A2 cocktail, was complexed with F127 and administered to 4^{CV} mice through intramuscular or intraperitoneal routes. Animals were sacrificed 3, 5 and 7 days after injection and RNA from the diaphragm and TA muscle was assessed for exon skipping. Although sporadic exon 52 and 53 skipping was detected in the TA muscle, no substantial dystrophin protein restoration could be demonstrated by either immunofluorescence or western blot analysis (data not shown).

Induced exon 52 and 53 skipping with phosphorodiamidate morpholino oligomers

We had previously found that, although 2OMeAOs were well suited for AO design and short-term *in vitro* RNA

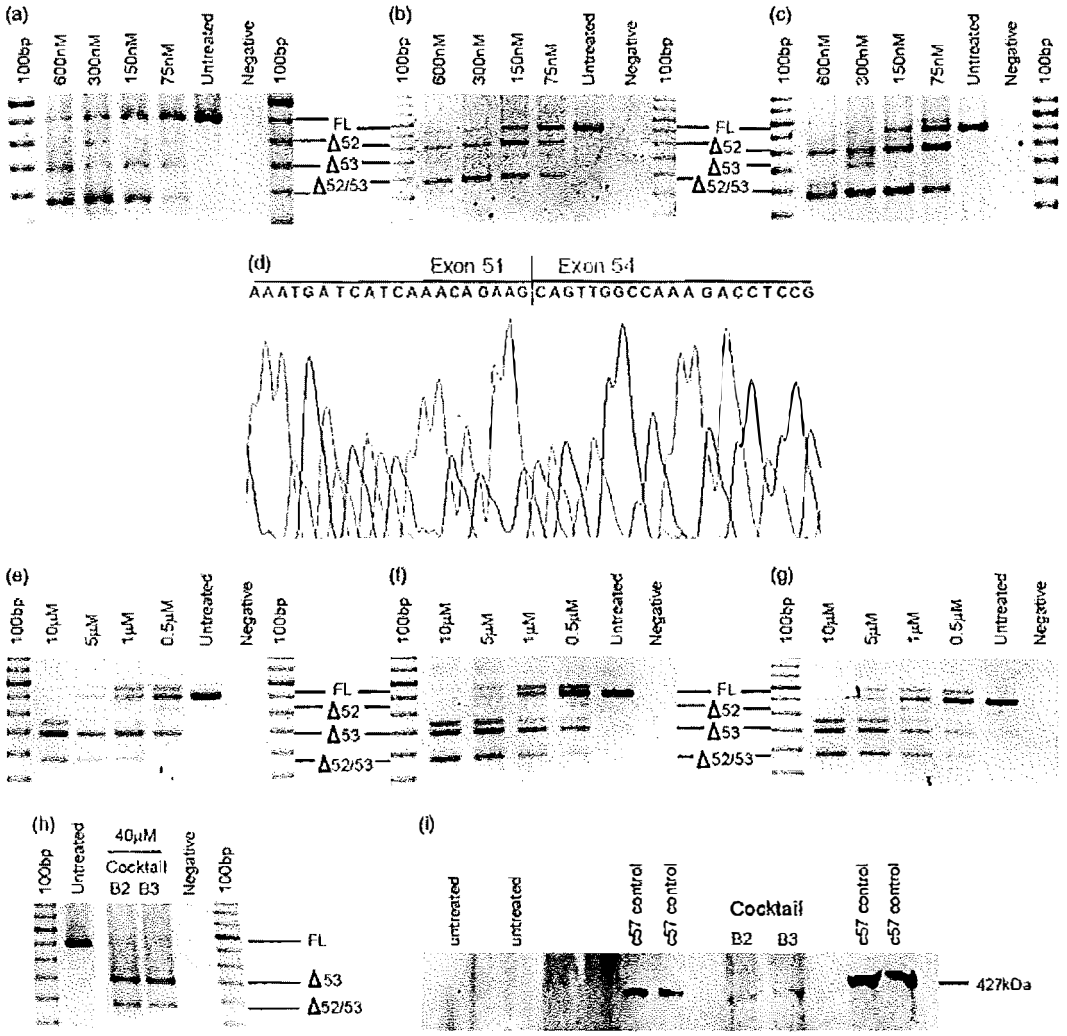

Figure 3. Induced exon skipping in 4^{CV} cells after transfection with 2OMeAO cocktails (a) A1, (b) A2 and (c) A3; (d) DNA sequencing chromatogram confirming precise splicing of exons 51–54. Transcripts resulting after transfection with (e) B1, (f, h) B2 and (g, h) B3 in 4^{CV} cultures; (i) western blot analysis from 4^{CV} cultures treated with B2 and B3 PMO cocktails

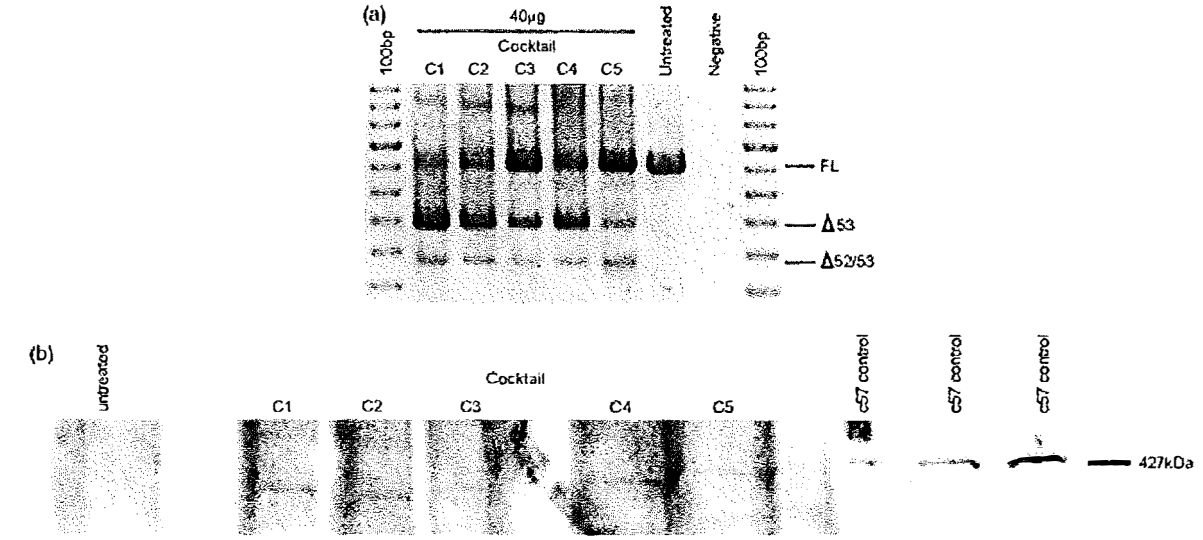

Figure 6. (a) RNA studies; and (b) western blot analysis of muscle extracts from the 4^{CV} mice injected (IM) with PMO cocktails (C1 to C5)

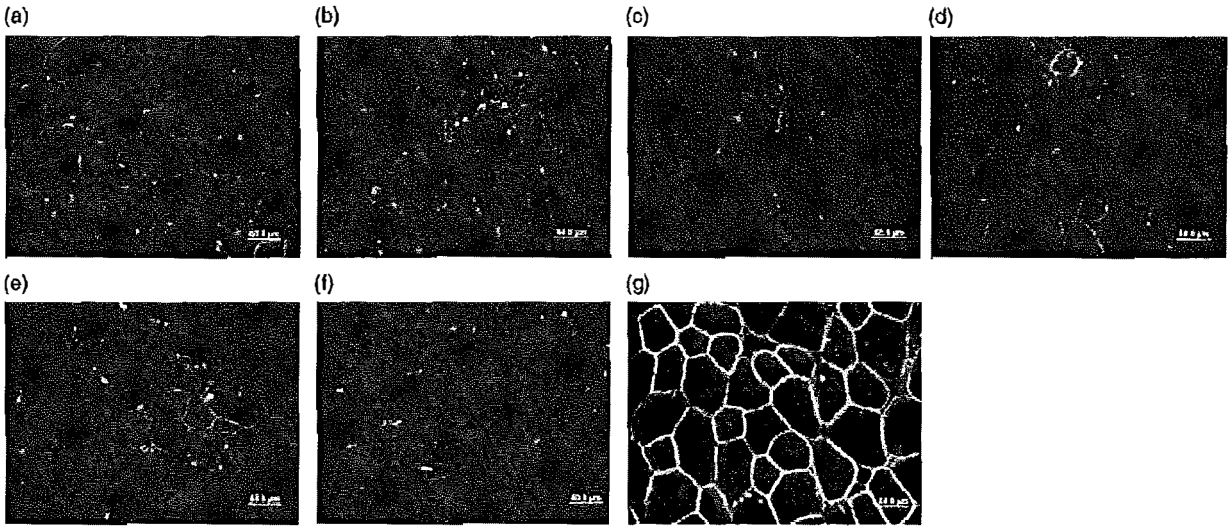

Figure 7. Dystrophin immunofluorescence on muscle cryosections from 4^{CV} mice treated with cocktails (a) C1, (b) C2, (c) C3, (d) C4, (e) C5 and (f) saline; (g) untreated C57

oligomer targeting the donor splice site [14]. However, a comprehensive study targeting all human dystrophin exons indicated donor splice sites are rarely the optimal or preferred targets, and the majority of preferred targets appear to be intra-exonic motifs [39]. Indeed, it appears that if a pre-mRNA site is not amenable to oligomer intervention, the application of a panel of oligomers to microwalk across that motif, or altering AO length or chemistry, is unlikely to achieve acceptable (or detectable) levels of exon exclusion [40]. Similar trends were observed for the 4^{CV} mouse dystrophin exons 52 and 53, where individual oligomers annealing to either the donor or acceptor splices sites failed to induce any targeted exon skipping. Two overlapping oligomers, AOs 1 and 4, targeting intra-exonic motifs within exon 52, were identified that induced substantial skipping and titration studies indicated AO 4 was slightly more efficient at lower transfection concentrations.

Mouse dystrophin exon 53 proved to be much more challenging to dislodge from the mature mRNA. Eighteen oligomers were designed to target acceptor and donor splice sites, as well as intra-exonic motifs predicted by ESEfinder [32,33]. Although this program is based upon human sequences, the strong homology between mouse and human dystrophin sequences was considered sufficient justification to use this program as a starting point in AO design. Initial AO design was undertaken in an immortalized *mdx* mouse myogenic cell line, while awaiting establishment of the 4^{CV} mice colony. The sequences of dystrophin exon 52 were identical in both strains, and similar identified splice patterns were generated with AO 4 in both cell lines. Similarly, AOs

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that led to exon 53 skipping in the *mdx* cell line exhibited identical patterns in cultured cells from the 4^{CV} mouse. Because no aberrant splicing was observed involving exon 53 in untreated 4^{CV} cultures, it was presumed that the mutation did not influence splicing. This is consistent with the mutation occurring in two putative ESEs that were just above the threshold for SF2/ASF and SRp40. Once the 4^{CV} colony was established, all subsequent experiments were undertaken in these cells.

The majority of compounds targeting exon 53, when used individually were either ineffective, activated a cryptic splice site (a loss of 78 bases from the end of exon 53) or led to the production of dystrophin gene transcripts missing exons 53 and 54, in addition to exon 53 alone (Figures 2d to 2f). Interestingly, AO 7, which anneals to exactly the same coordinates identified as an optimal target to dislodge human dystrophin exon 53, induced mouse dystrophin transcripts missing exons 53 and 54, in addition to exon 53 excision. Several other AOs overlapping AO 7 also induced this pattern of exon removal, implying that this area may be involved in coordinated processing of both exons during pre-mRNA splicing, at least in mice. Targeting exon 53 for excision from the human dystrophin gene transcript only resulted in specific target removal, highlighting some limitations in extrapolation of oligomer design between homologous genes of different species [39]. The loss of an exon flanking that targeted for excision has been reported previously. The AO targeting the *mdx* mouse exon 23 for removal also induced dystrophin gene transcripts missing exons 22 and 23, an out-of-frame transcript that cannot lead to dystrophin production [36]. Similarly, transcripts missing exons 53 and 54 are out-of-frame, even when exon 52 was omitted and were thus considered undesirable.

Because we had previously observed that some combinations of apparently inactive AOs were able to induce highly efficient exon skipping [41], various AO cocktails targeting exon 53 were evaluated. A combination of AOs 8 and 9, targeting the acceptor and donor sites, respectively, was able to induce some exon 53 skipping, although not at a consistent and satisfactory level. Subsequently, AOs 8 and 9 were combined with other AOs (7, 10, 11, 12, 13, 14 and 16) to identify a cocktail capable of inducing specific exon 53 skipping. When AO 13 was combined with AOs 8 and 9, efficient and specific exon 53 removal could be induced after transfection at low total AO concentrations. The mixture of AOs 8 and 13 generated the same pattern induced by AO 13 alone, indicating no advantage, whereas the combination of AOs 9 and 13 only induced inconsistent exon 53 excision. This three-AO combination was necessary for both efficiency and specificity of exon skipping.

Because exon 53 was removed from the mature mRNA at a higher efficiency than exon 52, attempts were made to balance the ratio of AO 4, targeting exon 52, with AOs 8, 9 and 13, to maximize induction of in-frame transcripts. When equimolar amounts of all four oligomers were applied, the full-length transcript was observed, in addition to dystrophin mRNAs missing exon 53 and exons 52 and 53. Altering the composition of the cocktails with increased proportions of AO 4 resulted in decreased amounts of full-length product and a higher proportion of in-frame transcripts missing both exons 52 and 53. Transcripts missing only exon 52, another out-of-frame mRNA were also observed.

Although a substantial amount of exon 52 and 53 deleted transcripts were detected at days 3 and 5 after transfection with 2OMeAOs, no detectable amount of dystrophin was observed by western blot analysis (data not shown). Only trace amounts of exon skipping could be observed 8–14 days after transfection, and this also did not generate sufficient dystrophin detectable by western blotting (data not shown). It was assumed that this limitation arises from the uptake and turn-over of the 2OMeAOs in cells. The 2′-*O*-modified oligomers on phosphorothioate backbone increase the oligomers resistance to nuclease degradation but, unlike PMOs, which are not metabolized, 2OMeAOs are gradually broken down by nucleases [42]. This was most apparent in a time-course of 2OMeAO-induced exon skipping in cultured cells, where there was substantial exon skipping 24 h after transfection but this declined substantially over the next 9 days [43]. By contrast, substantial PMO-induced exon skipping transcript had been detected with less intermediate product at 14 days after transfection, *in vitro* [44]. By-passing the dystrophin gene lesion and inducing dystrophin detectable by western blotting was very inconsistent in canine cultured cells from the golden retriever muscular dystrophy model of muscular dystrophy and human dystrophic muscle explants [35,44]. However, upon the application of PMOs of the same sequences, efficient exon skipping could be induced and maintained *in vitro* or *ex vivo* to allow sufficient dystrophin synthesis to be readily detected by western blotting at 7 days after transfection [35].

The application of any oligomer to a cell has the potential to cross react with other sequences and/or exert nonspecific effects. The use of AO cocktails would potentially increase this risk and, although a detailed examination of other gene has not been undertaken, we showed that the cocktail only induced targeted changes in the dystrophin mRNA. We had noticed in other studies that the addition of oligomers appears to marginally increase the appearance of alternatively spliced transcripts [45]. However, the application of four oligomers did not greatly alter the incidence of the alternative transcript in treated cells compared to untreated cultures.

The application of PMOs, coupled to an arginine rich cell-penetrating peptide, induced more robust exon skipping *in vitro* and *in vivo* in 4^{CV} cells than the equivalent 2OMeAOs. As we had previously shown that a hierarchy of exon skipping efficiency induced by 2OMeAOs was also seen when the same sequences were evaluated as PMOs [46], the most effective mixtures of 2OMe and PMO AO appeared comparable in the 4^{CV} model, although there was a minor shift in the relative proportions of exon excised transcripts. The optimal

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2OMeAO cocktails induced removal of approximately equivalent amounts of exons 52 and 52 and 53, whereas the equivalent PMO combinations generated transcripts missing exon 53 as the predominant transcript and exons 52 and 53. Approximately 10% of restored dystrophin protein was induced according to calculation of band densitrometry of western blot analysis.

PMO cocktails, consisting of a total of 40 μg of the oligomers directed to exons 52 and 53, were administered into the *TAs* of 4^{CV} mice by a single intramuscular injection. Two weeks later, dystrophin was observed by dystrophin immunofluorescence after C1, C2 or C3 treatment. Calculation of band densitometry from western blot analysis demonstrated approximately 5–10% of restored dystrophin protein after C1, C2 or C3 treatment. Further studies in this area may provide additional information to understand and to refine more efficient combinations for AO-induced dual exon skipping.

In summary, there still appears to be no consistent pattern in AO design for targeted exon skipping. Masking an obvious splice motif, such as the donor splice site that proved so effective in the *mdx* mouse, was ineffective in excising different exons in another mouse model of muscular dystrophy. AO cocktails were essential for by-passing the mutation in the 4^{CV} mouse in terms of both specificity and efficient exon removal. Consistent with other studies, the PMOs appeared to be superior to 2OMeAOs, especially *in vivo*. Long-term systemic treatment of the 4^{CV} mouse is now underway to establish an appropriate delivery regimen that would be best suited for dual exon skipping in a region of the dystrophin gene located within the human deletion hotspot.

Supporting Information

Supporting information may be found in the online version of this article.

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Rational Design of Antisense Oligomers to Induce Dystrophin Exon Skipping

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Duchenne muscular dystrophy (DMD), one of the most severe neuromuscular disorders of childhood, is caused by the absence of a functional dystrophin. Antisense oligomer (AO) induced exon skipping is being investigated to restore functional dystrophin expression in models of muscular dystrophy and DMD patients. One of the major challenges will be in the development of clinically relevant oligomers and exon skipping strategies to address many different mutations. Various models, including cell-free extracts, cells transfected with artificial constructs, or mice with a human transgene, have been proposed as tools to facilitate oligomer design. Despite strong sequence homology between the human and mouse dystrophin genes, directing an oligomer to the same motifs in both species does not always induce comparable exon skipping. We report substantially different levels of exon skipping induced in normal and dystrophic human myogenic cell lines and propose that animal models or artificial assay systems useful in initial studies may be of limited relevance in designing the most efficient compounds to induce targeted skipping of human dystrophin exons for therapeutic outcomes.

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INTRODUCTION

Duchenne muscular dystrophy (DMD), one of the most severe neuromuscular disorders of childhood, is caused by the absence of a functional dystrophin,[1] with patients suffering from progressive muscle weakness and severe respiratory and cardiac complications by the second and third decades of life.[2,3] Becker muscular dystrophy also arises from mutations in *dystrophin*, but these lesions are such that some functional protein can be generated, albeit of reduced quantity and/or quality.

Splice intervention using antisense oligomers (AOs) is being developed as a potential molecular therapy for DMD. AO intervention during dystrophin pre-mRNA processing aims to exclude one or more exons associated with the primary DMD-causing mutation, while maintaining or restoring the dystrophin mRNA reading frame. A clinical trial of AO-induced exon skipping in DMD patients has demonstrated proof of principle that this antisense strategy can restore some dystrophin expression in DMD muscle.[4]

One of the major challenges to oligomer-induced gene transcript manipulation for therapeutic purposes will be in the design and development of clinically relevant oligomers. Various models, including cell-free extracts, cells transfected with artificial constructs, mice with a human transgene, and *in silico* predictions, have been proposed as tools to facilitate oligomer design for splice manipulation.[5–7] The highly coordinated nature of gene expression leads to reduced efficiency of processing when cell-free extracts are used to assay splicing. Within a nucleus, introns are removed an estimated 40 times faster than *in vitro* processing of synthetic pre-mRNA transcripts.[8] Normal gene expression appears precariously balanced when one considers minor base changes can lead to activation of cryptic splice sites, pseudoexon inclusion, or some other form of aberrant splicing.[9,10] Hence, insertion of any test exon with some arbitrarily selected flanking intronic sequences into a splice reporter system may allow some assessment of oligomer-induced redirection of splicing patterns in transfected cells. However, such a system will not directly reflect processing of that exon when it is under control of the full complement of tissue specific *cis*- and *trans*-splicing motifs and factors. Finally, transcription-coupled processing differs from uncoupled processing in that the nascent pre-mRNA is a growing strand that is constantly folding into new structures and associating with protein–RNA and protein–protein complexes within the "mRNA factory."[11]

We consider normal human myogenic cells appropriate for AO design and optimization, and reported an initial draft of AOs targeting human dystrophin exons 2–78.[12] Despite strong homology between mouse and human *dystrophin*, we found variation in exon skipping efficiency and patterns of exon removal in these transcripts. For some exons, directing oligomers to the same target motifs induced similar exon skipping patterns. In contrast, oligomers targeting other exons generated different dystrophin splice isoforms in the two species. We report substantially different levels of exon skipping induced in normal and dystrophic human myogenic cell lines, and propose that animal models or artificial assay systems, useful in some studies, may be of limited relevance in designing the most efficient compounds to induce targeted skipping of human dystrophin exons.

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RESULTS

Splice site and auxiliary motif predictions

Acceptor and donor splice site scores for exons under investigation, as calculated by the web-based program, MaxEntScan are shown in **Table 1**, along with details of exon and flanking intron lengths. Although there was some variation in the mean donor splice site strength of those exons under investigation, there was no obvious trend where strong or weak donor splice sites were found to be more responsive as targets for splice intervention. Similarly, the score for the acceptor splice sites did not offer any indication as to the suitability of the sites for AO-induced exon skipping. It is of interest that the mouse dystrophin exon 23 acceptor splice site, predicted to be very weak, has previously been shown to be unresponsive as a target for exon removal.[13]

Exons and limited flanking intronic sequences were analyzed for exonic splice enhancers (ESEs), using two programs: ESEfinder 3.0 predicts motifs responsive to the human SR proteins SF2/ASF, SC35, SRp40, or SRp55,[14,15] and RESCUE-ESE allows identification of putative ESEs for human and mouse.[16,17] AO sequences designed to excise selected exons, and predicted ESEs, within these annealing sites are described in **Table 2**. The distribution of splice motifs predicted by RESCUE-ESE, relative AO annealing, and induced exon skipping patterns for both human and mouse dystrophin exons under investigation are shown in **Figure 1a–e**. The most effective AOs appear to preferentially target predicted SF2/ASF (IgM-BRCA1) (25%) or SC35 (28%) motifs, compared to 22, 17, and 8% targeting SF2/ASF, SRp40, and SRp55, respectively. These percentages are calculated from the number of ESE motifs, either completely or partially occurring within the oligomer annealing site, divided by the total number of ESE motifs within the target exons.

AO design and evaluation

A direct comparison of relative AO-induced skipping of the same exon in immortalized *mdx* mouse and human myogenic cells was undertaken to establish, if annealing coordinates for one species could be directly applied to the other. Immortalized *mdx* cells were assessed as removal of exon 23 would bypass the nonsense mutation, and exon 23 skipped transcripts would no longer be subjected to nonsense-mediated decay, unlike the intact, full-length transcript. Removal of other exons from the *mdx* dystrophin gene transcript would not remove the premature stop codon, hence both full-length and skipped transcripts should be subjected to equal rates of nonsense-mediated decay. Exclusion of selected exons from the normal dystrophin gene transcript will, in many cases, disrupt the reading frame and render the induced transcript subject to nonsense-mediated decay. In most experiments, dose responses of exon skipping with varying oligomer concentration were used to rank oligomer efficiency for that exon. A comprehensive series of AOs has been developed to excise each exon from the human dystrophin gene transcript.[12,18,19] Oligomer-induced exon skipping of several mouse dystrophin exons has been examined in normal and dystrophic mice, and is an on-going process of refinement.[20,21]

In 3 of 10 exons under investigation, optimal annealing coordinates for induction of exon skipping were identical in human

Table 1 Predicted acceptor and donor splice site scores showing length of exon and flanking introns

Exons	Preceding intron length	Exon length	Following intron length	3′ splice site	MEM[a]	MM[a]	5′ splice site	MEM[a]	MM[a]
H19	16,165	88	10,236	tctttgctctcatgctgcagGCC	9.1	9.24	ATGgtaatt	6.49	5.07
M19	17,035		8,906	cctttgctctcatgctgcagGCC	9.16	9.07	ATGgtaatt	6.49	5.07
H20	10,236	242	6,177	aattatttttttctttctagAGG	8.65	11.04	AAGgtaaga	10.57	10.55
M20	8,906		4,365	tatttggtttctttgtagAGG	7.82	10.51	AAGgtaagg	10.51	10.49
H21	6,177	181	12,609	ttccatactctatggcacagGAT	4.63	6.14	CAAgtaagt	10.08	9.07
M21	4,365		15,930	tactctgaattatgatgcagGAT	5.28	5.8	CAAgtaagt	10.08	9.07
H22	12,609	146	3,453	ttttttccctttgataaagTTT	4.79	5.43	CAGgtctgt	6.84	6.13
M22	15,930		913	tgttattctctttctttaagTTT	8.91	9.05	CAGgtctgt	6.84	6.13
H23	3,453	213	3,798	tttaaaaaatgtttttttagGCT	7.97	7.42	CAGgtaatt	8.55	7.72
M23	913		2,606	aacttctatttaatttttgagGCT	1.94	0.92	CAGgtaagc	9.88	11.18
H24	3,798	114	991	tataacgggtctcgtttcagAAT	8.84	6.45	AGAgtaaga	5.73	5.95
M24	2,606		1,096	atattgctttttttatttccagAAT	7.56	10	AGAgtaaga	5.73	5.95
H25	991	156	8,606	aaattgatttatttttcttagCTT	4.36	5.32	CAGgtatag	8.73	7.25
M25	1,096		5,983	actatgcattgtttccatagCTT	7.16	7.46	CAGgtatga	9.46	8.96
H52	44,211	118	50,044	agggatatttgttcttacagGCA	6.66	5.58	GAAgtaagt	9.82	8.09
M52	57,507		43,569	attttttttttttctttcagGCA	11.71	13.43	GAAgtaagt	9.82	8.09
H53	50,044	212	21,230	tatttttccttttattctagTTG	8.55	11.07	AAGgttagt	8.54	7.21
M53	43,569		23,325	tattcttatttttattccagTTG	7.81	10.24	AAGgttagt	8.54	7.21
H65	13,347	202	2,830	attttatttgtttttttgcagTGG	8.77	11.14	TACgtacgt	6.81	6.19
M65	15,253		2,234	ttgtgtggtctttttttgcagTGG	8.37	10.48	TACgtaagt	9.27	6.93

[a]MEM and MM are maximum entropy model and first-order Markov model, respectively.

Table 2 Nucleotide sequences of oligomers designed and evaluated for exon skipping potential

Exon	Sequence (5′→3′)	Coordinates	Size	SF2/ASF	SF2/ASF	SC35	SRp40	SRp55
H16	CUA GAU CCG CUU UUA AAA CCU GUU AAA ACA A	H16A(−12+19)	31	3.12(2)		2.45	4.05(3)	3.07
	GAU UGC UUU UUC UUU UCU AGA UCC G	H16A(+11+35)	25	2.24		2.49(2)		3.07
HM19	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U	HM19A(+35+65)	31	2.82(2)	3.34(3)	2.40(2)	2.77	
H20	CUG GCA GAA UUC GAU CCA CCG GCU GUU C	H20A(+44+71)	28	4.62(2)	4.18(3)	4.09(4)	2.83	
	CAG CAG UAG UUG UCA UCU GCU C	H20A(147+168)	22	3.82	3.20(2)	3.63(3)	4.57(2)	
	UUG GCA GAA UUC UGU CCA CCG GCU GUU C	M20A(+44+71)						
	CGG CAG UAG UUG UCA UCU GUU C	M20A(147+168)						
M20	GUU CAG UUG UUC UGA AGC UUG UCU G	M20A(+23+47)	25	4.62(2)	4.17(2)	2.68	3.88(2)	
	AGU AGU UGU CAU CUG UUC CAA UUG U	M20A(+140+164)	25	4.22(2)	4.18(2)	3.64(3)	4.56(3)	
	GUU CAG UUG UUC UGA GGC UUG UUU G	H20A(+23+47)						
	AGU AGU UGU CAU CUG CUC CAA UUG U	H20A(+140+164)						
H21	CUG CAU CCA GGA ACA UGG GUC C	H21A(+85+106)	22	3.87(2)	3.24(2)	4.81(3)	3.37	4.35
M21	CUG CAU CCA GAA ACA UUG GCC C	M21A(+85+106)		3.87(2)	3.25	4.7(3)	4.72(2)	4.35
H22	CUG CAA UUC CCC GAG UCU CUG C	H22A(+125+146)	22	2.23	2.61(2)	4.21(2)	3.90(2)	2.95
	CUG UAA UUU CCC GAG UCU CUC C	M22A(+125+146)						
M22	AUG UCC ACA GAC CUG UAA UU	M22D(+08-12)	20	2.68(2)	2.72	4.21	6.01(2)	3.41
	AUA UUC ACA GAC CUG CAA UU	H22D(+08-12)						
H23	CGG CUA AUU UCA GAG GGC GCU UUC UUC GAC	H23A(+69+98)	30	2.04	2.32(3)	3.66		
	UGG CAU AUU UCU GAA GGU GCU UUC UUG GCC	M23A(+69+98)						
M23	GGC CAA ACC UCG GCU UAC CUG AAA U	M23D(+07−18)	25	3.66(2)	3.9		4.97	
	AGU AAA AUC UUG AAU UAC CUG AAU U	H23D(+07−18)						
H24	CAA GGG CAG GCC AUU CCU CCU UC	H24A(+51+73)	23	2.79(2)	2.31(3)	2.67	2.98	
	CCA GGG CAG GCC AUU CCU CUU UC	M24A(+51+73)						
M24	CAA CUU CAG CCA UCC AUU UCU GUA A	M24A(+16+40)	25	2.34	2.45	3.02	3.63	3.74
	GAG CUG UUU UUU CAG GAU UUC AGC A	M24A(+78+102)	25			4.31(2)		
	CAA CUU CAG CCA UCC AUU UCU UCA G	H24A(+16+40)						
	CAG CUG CUU UUU UAG AAU UUC UGA A	H24A(+78+102)						
H25	UUG AGU UCU GUC UCA AGU CUC GAA G	H25A(+95+119)	25	2.29		3.16(2)		3.83
	CUA AGU UCU GUC UCC AGU CUG GAU G	M25A(+95+119)		3.50(2)	2.94	3.02	4.21	5.53
H52	UCC AAC UGG GGA CGC CUC UGU UCC AAA UCC	H52A(+12+41)	30	3.53(2)	2.74(4)	4.78(3)	3.55	3.33(2)
	UCC AAU UGG GGG CGU CUC UGU UCC AAA UCU	M52A(+12+41)						
M52	UCC AAU UGG GGG CGU CUC UGU UCC A	M52A(+17+41)	25	2.56	2.54(2)	3.33(3)		
	UUC AAA UUC UGG GCA GCA GUA AUG AGU UCU	M52A(+42+71)	30		2.73(2)	2.91(2)	4.92	3.02
	UCC AAC UGG GGA CGC CUC UGU UCC A	H52A(+17+41)						
	UUC AAA UUU UGG GCA GCG GUA AUG AGU UCU	H52A(+42+71)						
H53	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G	H53A(+39+69)	31	3.08(2)	3.69		3.19	
	CAU UCA ACU GUU GUC UCC UGU UCU GCA GCU G	M53A(+39+69)						
M53	CAG CCA UUG UGU UGA AUC CUU UAA CAU UUC	HM53A(+69+98)	30			2.94(2)	3.46(2)	
	UUU UAA AGA UAU GCU UGA CAC UAA CCU UGG	M53D(+05−25)	30		2.52	4.26	3.89	3.28(2)
	UUA AAA AGG UAU CUU UGA UAC UAA CCU UGG	H53D(+05−25)						
	CUA CUG UGU GAG GAC CUU CUU UCC AUG AGU	M53A(+151+180)	30	3.86(3)	3.65(3)	4.58(2)	3.64(3)	
	UCU GUG AUC UUC UUU UGG AUU GCA UCU ACU	M53A(+176+205)	30	3.41	3.41	4.03	3.94(2)	2.91
H65	GCU CAA GAG AUC CAC UGC AAA AAA C	H65A(−11+14)	25	3.29	3.15	2.52(2)	3.01	
	GUU GUG CUG GUC CAA GGC AUC ACA U	H65A(+26+50)	25			3.61	4.35	3.76
	GCU CAA GAG AUC CAC UGC AAA AAA G	M65A(−11+14)						
	GUU GUG CUG GUC CAG GGC AUC ACA U	M65A(+26+50)						
HM65	UCU GCA GGA UAU CCA UGG GCU GGU C	HM65A(+63+87)	25	2.77	2.97(3)	3.42(2)	3.20	4.35

Oligomers for the human and mouse dystrophin pre-mRNA are grouped by exon, dotted lines separate individual antisense oligomers (AOs) from AO cocktails. Maximum exonic splice enhancer (ESE) binding scores, as predicted by ESE finder, are shown with the number of ESEs above the threshold occurring within the AO annealing site indicated in brackets. SF2/ASF and SF2/ASF(IgM-BRCA1) predictions are based upon functional SELEX experiments to examine effects of sequence context on motif derivation.[14]

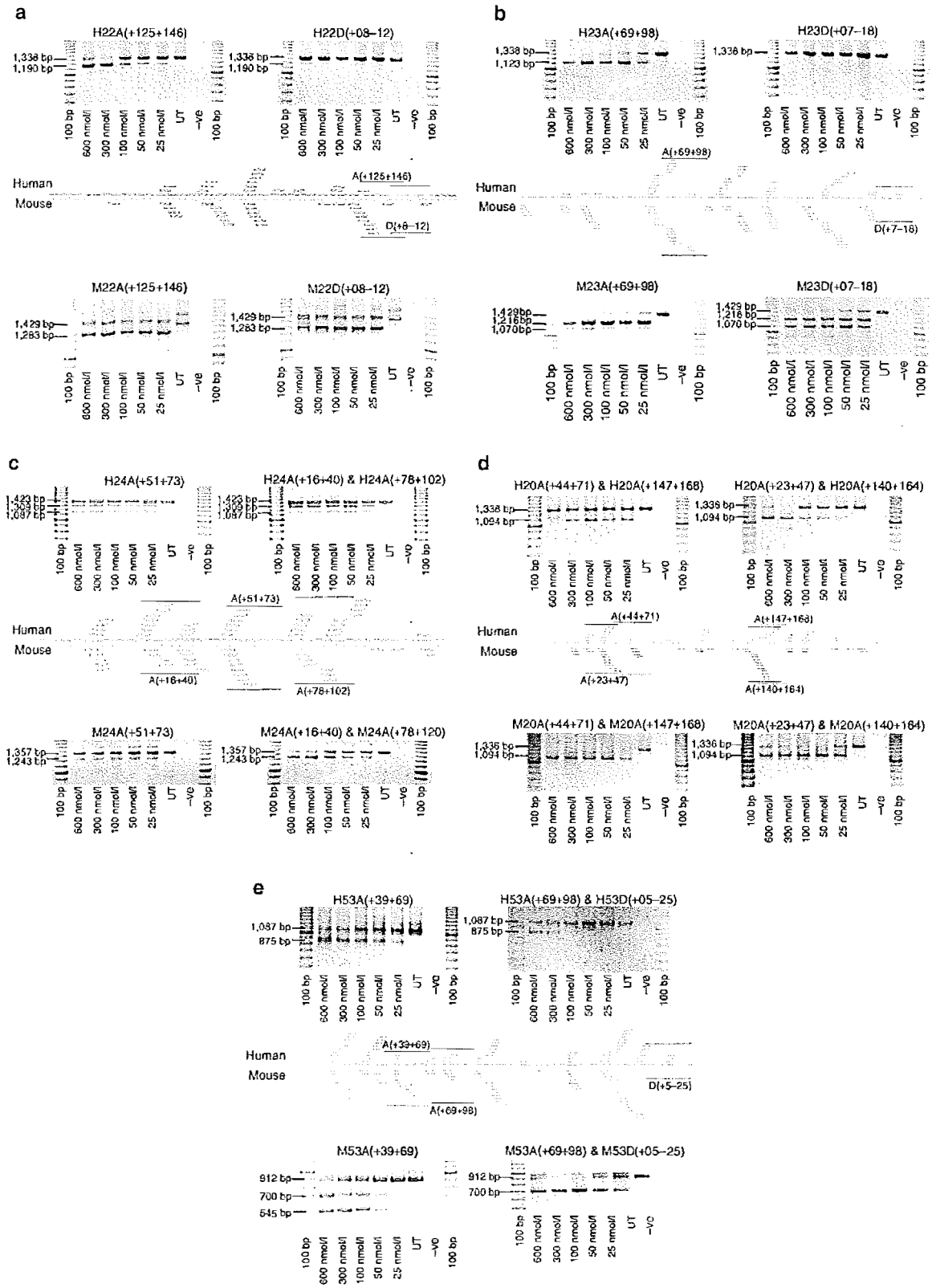

Figure 1 Comparison of exon skipping induced in human and mouse myogenic cells. Patterns of dystrophin exon skipping induced by antisense oligomers (AOs) targeting 5 exons [(**a**) 22; (**b**) 23; (**c**) 24; (**d**) 20; (**e**) 53] after transfection into normal human and *mdx* mouse myogenic cells. Annealing coordinates relative to predicted exonic splice enhancers are shown, as are the sizes of full-length and induced transcript products. UT, Lipo, or L2K refer to untreated cells, or cells treated with Lipofectin, or Lipofectamine 2000, respectively.

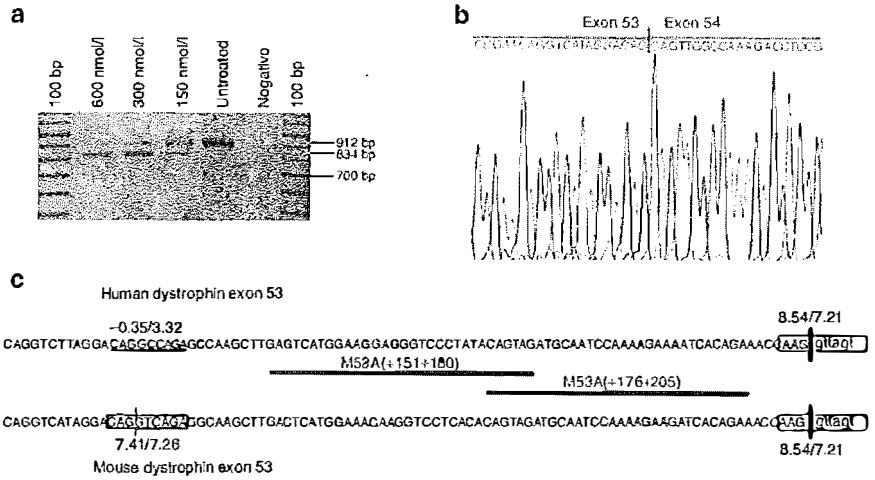

Figure 2 Antisense oligomer (AO)-induced cryptic splicing of mouse dystrophin exon 53. (**a**) Dystrophin transcript products in AO-treated *mdx* cultures; (**b**) nucleotide sequences at the novel junction arising from activation of the exon 53 cryptic donor splice site identified by DNA sequencing; (**c**) partial sequence of human and mouse dystrophin exon 53 showing mouse cryptic donor splice site and AO annealing coordinates. Differences in nucleotide sequences are indicated in bold type.

and mouse, indicating that transfer of AO design between human and mouse dystrophin exons 19, 21, and 25 was possible (data not shown). As shown in Table 1, the calculated acceptor splice site scores for exon 19 was high (9.07–9.24), while the donor splice site scores were moderate (5.07–6.49). In contrast, the scores for exon 21 acceptor (4.63–6.14) were much weaker than the donor (9.07–10.08), whereas the acceptor and donor site scores of exon 25 were of intermediate values (4.36–7.46 and 7.25–9.46, respectively).

Exons 19, 21, and 25 were removed by single AOs, as were exons 22 and 23, although in the latter cases there were substantial differences in exon skipping efficiency and splicing patterns between the species. For both mouse exons 22 and 23, the donor splice sites were originally identified as amenable targets for exon excision, however, when these coordinates were targeted in the human dystrophin pre-mRNA, there was no detectable exon skipping (**Figure 1a,b**). Targeting intraexonic motifs in human dystrophin exons 22 and 23 induced substantial exon skipping, and directing AOs to these coordinates in the mouse dystrophin pre-mRNA also resulted in efficient exon excision. Indeed, it would appear that directing an oligomer to the corresponding mouse intraexonic motif identified in human dystrophin exon 22 was equally effective at excising the target exon, as the oligomer annealing to the donor splice site. The induced splicing patterns for exon 23 also differed between human and mouse, in that when human exon 23 removal was specific (1,216 bp), transcripts missing exons 22 and 23 (1,070 bp) were readily detected in the treated mouse cells, in addition to specific exon 23 skipping. This dual exon skipping was more pronounced when the mouse exon 23 donor site was targeted, and it would appear that directing an oligomer to the intraexonic motif may result in more specific exon 23 skipping.

Human exon 24 could be excised with a single AO, H24A(+51+73) (1,309 bp), but reverse transcription–PCR (RT-PCR) also revealed the presence of transcripts missing exons 24+25 (1,087 bp). Targeting the equivalent mouse coordinates resulted in lower levels of exon 24 skipping in an apparent nondose-dependant manner. More efficient and specific mouse exon 24 skipping was induced using a combination of two AOs. The application of an AO cocktail targeting the equivalent coordinates induced skipping of human exon 24, but transcripts missing exon 24 and 25 were still evident (**Figure 1c**).

Two of the 10 human exons (20 and 65)[22] and 4 of the 10 mouse exons (20, 24,[21] 52, and 53[20]) were only efficiently dislodged by the application of combinations of AOs. There was partial overlap in AO annealing coordinates for the cocktails that excised exon 20 from both the human [H20A(+44+71) and H20A(+147+168)] and mouse [(M20A(+23+47) and M20A(+140+164)] dystrophin gene transcripts (**Table 2**).[12,21,22] The human and mouse AO cocktails appeared equally efficient when used in mice, but when the optimal mouse coordinates were directed to the human dystrophin pre-mRNA, there was a slight, but reproducible decline in efficiency of exon removal, most noticeable at lower transfection concentrations (**Figure 1d**).

A single AO, H52A(+12+41), was found to efficiently remove human exon 52 from the mature dystrophin transcript, with >30% exon skipping being induced after transfection at 100 nmol/l.[12] Directing an oligomer to the corresponding mouse coordinates induced substantial exon skipping, but the application of the cocktail M52A(+17+41) and M52A(+42+71) resulted in two- to threefold more exon exclusion (**Supplementary Figure S1**).

Directing AOs to human and mouse dystrophin exons 53 resulted in the generation of the most distinct patterns of exon excision observed to date. A single AO, H53A(+39+69), was able to induce efficient and specific exon skipping from the human dystrophin gene transcript (**Figure 1e**). Upon targeting the same coordinates in the mouse dystrophin pre-mRNA, some transcripts missing exon 53 (700 bp) were detected, as well as a substantial proportion of transcripts missing both exons 53 and 54 (545 bp). The shorter transcript is out-of-frame and was never detected after transfecting human cells with this AO or any of the other 18 AOs, designed to excise human exon 53.[20] Specific mouse exon 53 skipping could be induced using a combination of two mouse-specific

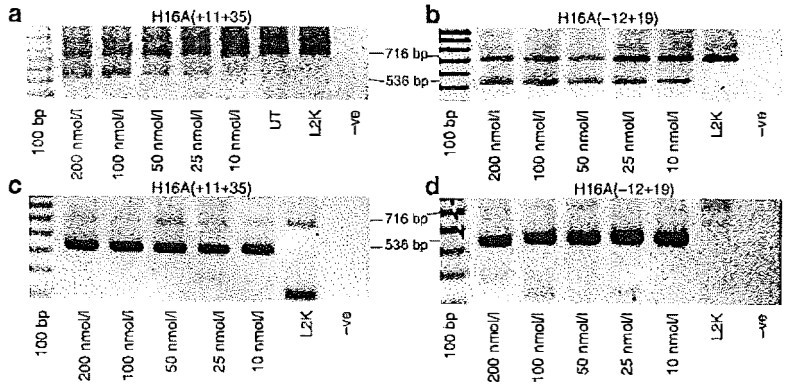

Figure 3 Comparison of exon skipping induced in normal and patient myogenic cells. Reverse transcription–PCR analysis of two oligomers to induce exon 16 skipping in normal myogenic cells (**a** and **b**); and cells from a Duchenne muscular dystrophy patient with a mutation (IVS16+1G>T; c.1992+1G>T) in dystrophin exon 16 (**c** and **d**). UT or L2K refer to untreated cells, or cells treated with Lipofectamine 2000.

AOs (**Figure 1e**). However, when the same annealing coordinates were targeted in the human dystrophin gene transcript, only low levels of exon 53 skipping were detected (**Figure 1e**).

Two additional AOs, [M53A(+151+180) and M53A(+176+205)], with overlapping annealing sites, were found to induce cryptic splicing in mouse exon 53, which led to the loss of 78 bases of coding sequence from 3′ end of the exon (**Figure 2a**). The activated mouse cryptic donor splice site was identified by DNA sequencing (**Figure 2b**), and calculated to have a splice site score of 7.41/7.26 (**Figure 2c**). Directing oligomers to this region of the human gene transcript resulted in low levels of inconsistent exon 53 skipping, with no evidence of cryptic splice site activation (data not shown).

Human dystrophin exon 65 could only be efficiently removed using a combination of two AOs,[22] whereas AOs, M65A(−11+14) and M65A(+26+50), annealing to the same coordinates as the human cocktail components were able to induce robust exon skipping from the mouse dystrophin pre-mRNA, after transfection at concentrations of 300 nmol/l, but efficiency declined at lower concentrations (**Supplementary Figure S2**). Another oligomer targeting mouse exon 65, M65A(+63+87) (shown in **Table 2**), was found to induce more robust exon skipping (**Supplementary Figure S2**). However, directing an oligomer to the corresponding coordinates in the human dystrophin pre-mRNA did not induce skipping of the target exon, when applied individually.[22]

Induced exon 16 skipping in normal and dystrophic human myogenic cells

Two AOs, H16A(−12+19)[12,23] and H16A(+11+35),[23] previously designed to excise exon 16 from the mature human dystrophin mRNA, could be readily distinguished in their ability to excise the target exon (**Figure 3a,b**). H16A(−12+19), targeting the exon 16 acceptor site could induce pronounced exon excision at a concentration of 10 nmol/l, while H16A(+11+35) induced weaker exon 16 skipping after transfection at 25 nmol/l in normal human myogenic cells. However, when these compounds were transfected into myogenic cells from a DMD patient with a G>T substitution of the first base of intron 16 (IVS16+1G>T; c.1992+1G>T), both AOs induced robust exon skipping and could not be readily discriminated (**Figure 3c,d**). RT-PCR on RNA extracted from the untreated DMD cells did not yield a consistent pattern, with sporadic generation of shorter than normal transcripts missing exons 13–15, 14–16, 14+16 (exon 15 present), and 16, and less abundant, near-normal, or larger than expected products. Some of the products of abnormal size were identified by DNA sequencing as having arisen from displacement of the exon 16 donor splice site by one base upstream, or pseudoexon inclusion of 89 bases from intron 16 (data not shown).

DISCUSSION

Pre-mRNA splicing, the process of joining exons and removing intervening sequences, is tightly controlled by complex interactions between *cis*-elements and >150 *trans*-factors[24–27] involved in recognition of exon boundaries and subsequent incorporation into the mature transcript. Four classical *cis*-elements; the 5′ and 3′ splice site, the polypyrimidine tract, and branch point are primary binding sites for snRNPs and other proteins involved in defining exon/intron boundaries. In addition to the obvious splice motifs, exon recognition and splicing also depends upon the nature, position, and combination of auxiliary splice motifs that modulate signals determining exon incorporation, presumably by recruiting *trans*-factors that regulate exon selection.[28,29]

The high degree of conservation at the ends of the introns would suggest that the acceptor and donor splice sites are obvious and preferable targets for AO-induced exon skipping. As all dystrophin exons are constitutively expressed in the predominant 427 kd skeletal muscle isoform, splice site scores are somewhat redundant, but may provide an indication of amenable targets to redirect pre-mRNA splicing. However, considering the 10 exons examined in this report, there was no obvious correlation between predicted splice site score and optimal AO target. Donor splice sites seemed to be the least preferred human targets, although these motifs were amenable targets in skipping 3 of the 10 mouse exons.

AO-induced exon skipping requires appropriately designed AOs to specifically mask motifs involved in the exon recognition and splicing process, by either rendering the single-stranded motifs double-stranded or altering the secondary structures essential for normal exon recognition and processing. Robust and consistent exon skipping is essential if oligomers are to be clinically

applicable. If it is necessary to use large amounts of an oligomer to excise a target exon, this may be difficult to achieve in a clinical setting, and/or lead to nonantisense effects. Although 12 oligomers may be capable of restoring the dystrophin reading frame in DMD individuals with exon deletions in the mutation hotspots,[30] it will be essential to extend this therapy to many different amenable mutations across the gene transcript.

Subtle DNA changes in *dystrophin* (microinsertion/deletion, nonsense, splice site mutations) represent an estimated 30% of DMD cases. These mutations cannot be ignored based only upon incidence, especially because the gene is largely intact and in most of these cases splice intervention would result in a dystrophin isoform of near-normal function. Furthermore, protein-truncating mutations in the exons encoding the rod domain could be considered most amenable to exon skipping. The majority of exons in this region are in-frame and hence removal of a nonsense mutation/microinsertion/deletion would only require excision of a single exon. In-frame deletions within this region are not commonly reported, possibly because many of these cases are not recognized due to a mild phenotype.[31] Indeed, it has been reported that an individual missing exon 16 had no clinical symptoms and normal serum creatine kinase levels, a sensitive marker of muscle damage.[32]

The 45 AOs evaluated for both human and mouse dystrophin pre-mRNA annealed to a total of 174 predicted ESEs. SF2/ASF (IgM-BRCA) and SC35 were found to be more common targets for the optimized AO-induced skipping of these dystrophin exons, consistent with other reports.[22,33] The high proportion of AOs capable of redirecting splicing supports the concept that many pre-mRNA motifs are involved in exon definition and splicing (for review see refs. 11,34,35). It is possible that the importance of some motifs in pre-mRNA processing is reflected by the levels of induced shortened transcripts. It should also be noted that no single motif is a universal optimal target. Mouse exons 22 and 23 were efficiently excised by targeting two distinct domains, the donor splice sites and intraexonic motifs, which were identified during a study of the human gene transcript. Although the human dystrophin gene transcript only responded to AOs directed at intraexonic targets for these exons, it is important that, in the event of sequence-specific effects with one compound, another oligomer may be available. In addition, if polymorphisms or disease-causing mutations compromise annealing of one oligomer, it will be necessary to have identified an alternative compound.

Although some AO coordinates appeared to be equally amenable in the two species, there were differences, some subtle and others more pronounced, that might raise questions regarding the accuracy and validity of AO design in nonhomologous or artificial systems. Directing an AO at coordinates found to efficiently excise exon 53 from the human dystrophin mRNA induced dystrophin gene transcripts missing exons 53 and 53 + 54 when applied to mouse cells. There are several cases where a single AO can excise two exons at a time, presumably reflecting closely coordinated processing.[30,36,37] Targeting human exon 53 resulted in only the loss of that exon from the mature dystrophin transcript, but interestingly, directing an AO to human dystrophin exon 54 led to removal of both exons 54 + 55.[12] An AO designed to excise mouse exon 54 resulted in specific removal of that targeted exon (C. Mitrpant, S. Fletcher, and S.D. Wilton, unpublished results). Although similarities in human and mouse dystrophin splice motif usage were predicted to be as high as 90% in constitutive splicing,[38–40] differences in genetic background and splice motif usage must be considered when extrapolating transcript manipulation from one model to another.

During initial optimization studies to induce mouse exon 53 skipping, two overlapping AOs were found to activate a cryptic donor splice site in that exon, which led to the loss of 78 bases of coding sequence. The activated mouse exon 53 cryptic splice site was calculated to have a splice site score similar to the wild-type donor site. When targeting the same coordinates in the human dystrophin transcript, activation of cryptic splicing was never detected. A "T" (mouse) and "C" (human) difference allowed activation of a cryptic donor splice site only in the mouse transcript, thereby demonstrating that species-specific variation, even when not directly affecting AO annealing, can have indirect and unexpected consequences on gene expression.

We consider it important to initially optimize AO design in myogenic cells expressing a normal dystrophin gene transcript, even though protein studies are not possible. Induced exon removal from the intact dystrophin mRNA would ensure that the intervention was possible in the presence of all normal transcription and splicing *cis*-elements and splicing machinery, thus setting a high standard in AO design. Exonic deletions that disrupt the reading frame and lead to DMD would compromise pre-mRNA processing to some extent, because of the loss of splice motifs in the deleted region. A deletion of exon 50 and flanking intronic sequences will bring together exons 49 and 51 that should not be in direct communication in the context of normal dystrophin transcript processing. Furthermore, a normal dystrophin gene transcript would not be subjected to nonsense-mediated decay, unlike induced transcripts in which exon removal causes a frameshift.[34]

We previously reported optimization of AOs to excise exon 16 from the human gene transcript.[23] The addition of five nucleotides to the AO was found to increase efficiency of target exon skipping by ~40-fold, more than justifying a 20% increase in the length of the oligomer. The exon 16 donor splice site mutation did not lead to a single aberrant gene transcript, but a mixture of products, including shorter in-frame transcripts that should have mitigated severity of the disease. Since the diagnosis of DMD had been confirmed clinically, it is most likely that the in-frame products represent very low abundance mRNAs that had escaped NMD and were, hence, detected by RT-PCR. A nonsense mutation in the muscle glycogen phosphorylase gene was reported to result in seven different gene transcripts generated by altered splicing.[41] While it appears that the exon 16 dystrophin donor splice site mutation generated multiple disease-associated transcripts, the AO that excised exon 16 restored apparently normal levels of a single in-frame transcript.

The evidence that single base variation between mouse:human or human:human *dystrophins* can influence splice manipulation must cast some doubts on assays that do not assess dystrophin splicing in the appropriate environment. It is unreasonable to assume that *in vitro* gene expression in a monolayer of cultured myogenic cells will exactly reflect *in vivo* gene expression, especially considering fiber-type differences, muscle architecture, innervation, and degree of pathology in dystrophic muscle. However, dystrophin

processing in cultured myogenic cells is likely to be more relevant than an artificial system examining transgene expression in a cell line transfected with a plasmid construct containing only a portion of *dystrophin* taken out of context. Goren *et al.*[40] demonstrated that the same auxiliary splice motif could direct either exon inclusion or exclusion from different mini genes, depending on the location of splice motifs in the construct. When sequences flanking splice regulatory motifs are manipulated, splicing is modified.[40] In such an artificial system, irrelevant intron size and sequences must be a major concern for AO design and optimization.

In summary, the design and evaluation of AOs to induce human dystrophin exon skipping should be undertaken in human myogenic cultures, whereas a study of exon excision from the mouse dystrophin gene transcript should be undertaken in murine cells. Despite strong homology between the human and mouse (and canine) *dystrophin*, there are many differences and some, as described in this report, influence RNA processing. When developing exon skipping strategies for nondeletion DMD patients, AOs designed according to the normal *dystrophin* may not be ideal. The disease-causing base change, loss, or insertion may occur at the AO annealing site and compromise its ability to excise that exon. Although it is possible that the change may alter a splice motif and enhance exon recognition and strengthen splicing, it is more probable that most changes in a gene would weaken exon recognition and splicing. Regardless of the predicted consequences of a change in dystrophin splicing, testing should be undertaken in myogenic cells, then dystrophic cells carrying the mutation under examination, and ultimately the patient.

MATERIALS AND METHODS

Splice site scoring and prediction of ESEs motifs. Acceptor and donor splice site scores for all exons under analysis were determined using two different algorithms; the maximum entropy model and the first-order Markov model. Both algorithms were computed on the web-based program, MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxent.html), which allows the calculation of strength of both acceptor and donor splice site scores. MaxEntScan requires 20 bases upstream of the 3′ splice site and the first three bases after the acceptor to perform 3′ splice site scoring. Three bases upstream and six bases downstream of the donor splice site were included to evaluate 5′ splice site scoring.[41] Exonic sequence with 25 bases of flanking intron from 10 human and mouse exons were analyzed to identify putative ESEs, using ESEsfinder3.0[14,15] for human exons, and human and mouse ESE motifs were predicted by RESCUE-ESE.[16,17]

AO synthesis, design, and nomenclature. 2′-O-methyl-modified AOs on a phosphorothioate backbone were designed to anneal to motifs predicted to be involved in pre-mRNA splicing, and synthesized in-house on and Expedite 8909 Nucleic Acid Synthesizer (Applied Biosystems, Foster City, CA), using the 1 μmol thioate synthesis protocol. Nomenclature was based on that described by Mann *et al.*,[37] where the first letter designates the species (H: human, M: mouse), the number refers to the exon, the second letter indicates Acceptor or Donor Splice site and the "−" and "+" specifies intron or exon bases, respectively.

Myoblast culture and transfection. Normal and dystrophic human myogenic cells were prepared by a modification of the protocol described by Rando and Blau.[43] Myogenic cells were transfected with AOs at concentrations of 25–600 nmol/l. Lipofectamine 2000 (Invitrogen, Melbourne, Australia) and Lipofectin (Invitrogen) were employed as transfection reagents for human myogenic cells and *H-2K mdx* cells, respectively, as described previously.[18]

RNA extraction and RT-PCR analysis. RNA was harvested from the cell cultures 24 hours after transfection, using Trizol (Invitrogen), according to the manufacturer's protocol. One-step RT-PCR was undertaken, using 100 ng of total RNA as template in a 12.5 μl reaction for 30 cycles, using 1 U of Superscript III (Invitrogen). Nested PCR was carried out for 30 cycles, using AmpliTaQ Gold (Applied Biosystems, Melbourne, Australia). PCR cycling conditions were performed, as described by McClorey *et al.*[36] PCR products were separated on 2% agarose gels in Tris–acetate–EDTA buffer and the images were captured on a CHEMISMART-3000 gel documentation system (Vilber Lourmat, Marne-la-Vallee, France).

SUPPLEMENTARY MATERIAL

Figure S1. (a) Exon 52 skipping induced by M52A(+12+41) and an AO cocktail of M52A(+17+41) & M52A(+42+71) at different concentrations in *H-2K mdx* myoblasts; and (b) the efficiency of exon skipping as determined by densitometric analysis of RT-PCR.

Figure S2. Exon 65 skipping induced by M65A(+63+87) and an AO cocktail of M65A(-11+14) & M65A(+26+50) at different concentrations in *H-2K mdx* myoblasts.

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