The potential of antisense oligonucleotides as a therapy for Duchenne muscular dystrophy in human and canine models of the disease

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked, fatal, muscle wasting disorder that causes boys to lose ambulation in their early teens, with death in the third decade of life. Despite identification of the gene responsible twenty years ago, there has been no cure for this debilitating disease, with current treatments focusing on prolonging muscle function without addressing the underlying genetic defect. DMD is caused by mutations in the DMD gene that precludes the synthesis of functional dystrophin, a structural protein that provides stability to the muscle during contraction. A recently proposed therapeutic strategy has been the use of antisense oligonucleotides (AOs) to manipulate pre-mRNA splicing to remove exons from the dystrophin transcript such that the DMD mutation is removed or bypassed to allow a semi-functional protein to be produced.

The majority of DMD research to date, has utilised the mdx mouse model, which exhibits a relatively mild dystrophic phenotype. In contrast, a canine model, the Golden Retriever Muscular Dystrophy or GRMD model, has a much more severe phenotype with pathological similarities to that observed in DMD patients. This body of work addresses the hypothesis that AOs could be used to correct the dystrophin mutation in this more clinically relevant model and hence provide an alternate model for optimising this approach prior to application to the human condition. To this end, AOs were developed to correct the dystrophin mutation both in vitro and in vivo, such that near-full length dystrophin protein could be detected. Various AO chemistries were also simultaneously tested for their ability to induce dystrophin to provide further knowledge of the optimal treatment using this approach.

The other aspects of this thesis focused on application of this AO exon skipping therapy to human models of DMD. The limits of this approach were tested by inducing multiple exon removal so as to potentially address numerous DMD mutations within the same region using only a single “cocktail” of AOs. Whilst successful for small multi-exon regions, larger regions were difficult to excise, which may reflect intrinsic limitations to how much splicing can be manipulated, or alternately may require further optimisation of AO design. Finally, muscle explants were used as an ex vivo model of DMD to demonstrate AO-induced exon skipping.
within the context of both normal and DMD patient tissue. This provides proof-of-principle that this approach can be successful in clinical trials, and is further encouragement that this approach will be beneficial to DMD patients in the near future.
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1.1 Dystrophin protein and DMD

1.1.1 Duchenne muscular dystrophy phenotype

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular disorder characterised by progressive muscle-wasting and weakness of variable distribution and severity (Emery, 1993). It is the most prevalent of the muscular dystrophies with an incidence of approximately 1 in 3500 male births (Moser, 1984). Onset of symptoms occurs in early childhood with difficulties in running and climbing stairs, and onset of the Gower’s manoeuvre, where due to weakness of the knee and hip extensors, a child uses his hands to push down on his thighs to rise from a sitting position (Emery, 2002). As the weakness progresses, boys will usually lose ambulation by the age of 12 and in the absence of treatment will die in their late teens and early twenties (Emery, 2002). Death commonly occurs due to respiratory illness compounded by cardiac involvement. Recent advances in respiratory management, in particular night-assisted ventilation (Mellies et al., 2003) has improved quality of life and life expectancy, such that some patients live into their late twenties or early thirties (Eagle et al., 2007). Some degree of mental impairment is also observed, with approximately 20% of patients having an IQ of less than 70 (Emery, 2002).

1.1.2 Identification of the DMD gene

Identification of the gene responsible for DMD was one of the first successes of positional cloning. Given that the majority of patients were male, DMD was proposed to be an X-linked disorder, and this was confirmed in studies of rare female DMD patients who had chromosomal translocations with breakpoints in the Xp21 region (Boyd and Buckle, 1986). Localisation to this region was further confirmed using DNA markers (Davies et al., 1983) and the gene was identified in a patient with a microscopically visible micro-deletion who suffered from four X-linked phenotypes, including DMD (Francke et al., 1985). The gene protein product was subsequently named dystrophin because its absence is the cause of the dystrophy (Koenig et al., 1987).
1.1.3 Becker muscular dystrophy

Interestingly, Becker muscular dystrophy (BMD) was localised to the same gene (Kingston et al., 1984). BMD affects approximately 1 in 30,000 males with a clinical course similar to DMD, although with later onset and delayed progression (Emery, 2002). Generally, age of onset is 12 years with loss of ambulation in the third or fourth decade and death between 40 and 50 years of age, however phenotypes can vary significantly from very mild to moderately severe (Emery, 1993). Approximately half of all BMD patients exhibit a cardiomyopathy and for some, this is the main symptom (Emery, 1993). Unlike in DMD, where there is negligible dystrophin, protein can be detected in BMD patients although it is of abnormal size and/or of reduced quantity (Hoffman et al., 1988). It was proposed that the difference in dystrophin levels between DMD and BMD patients was related to the nature of the dystrophin gene mutation. Deletions that caused a shift in the open reading frame would induce premature stop codons that would produce a truncated, non-functional protein that would result in a severe DMD phenotype. In contrast, deletions in BMD patients do not disrupt the reading frame, therefore internally deleted, yet semi-functional dystrophin proteins (as they retain the crucial amino- and carboxy-terminal domains) reduce the severity of disease (Monaco et al., 1988). This is known as the reading-frame hypothesis (Figure 1.1) and studies of dystrophin mutations reveal that this holds true for over 90% of DMD and BMD patients (Baumbach et al., 1989, Koenig et al., 1989).

**Figure 1.1 (A)** Normal splicing of 79 exons induces a full-length dystrophin protein that acts as a structural girder between cytoskeletal actin and the extracellular matrix. **(B)** In DMD, disruption of the mRNA reading frame (e.g. deletion of exon 45) results in a downstream premature stop codon, such that a truncated dystrophin protein is produced, with subsequent loss of function. **(C)** In BMD, an in-frame deletion (e.g. exon 45-46) allows synthesis of an internally deleted dystrophin that retains the amino- and carboxy- terminals essential for dystrophin function.
A Unaffected individual

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Full-length dystrophin

- pre-mRNA
- splicing
- mRNA
- translation
- protein

extracellular matrix

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Full-length dystrophin
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Figure 1.1 The reading-frame hypothesis

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B Duchenne muscular dystrophy

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Premature termination codon

- pre-mRNA
- splicing
- mRNA
- translation
- protein

extracellular matrix

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Truncated dystrophin
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C Becker muscular dystrophy

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In-frame

extracellular matrix

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Internally deleted dystrophin
```

Figure 1.1 The reading-frame hypothesis
1.1.4 Dystrophin protein

The muscle isoform of dystrophin is a 427kDa protein composed of four distinct domains (Figure 1.2). The amino terminal domain consists of two calponin homology motifs that form two actin binding sites (Ervasti and Campbell, 1993). The large central domain consists of 24 helical spectrin-like repeats, separated by 4 hinge domains which are thought to confer flexibility to this rod domain (Koenig and Kunkel, 1990). Within this domain an additional actin-binding site has been identified (Rybakova et al., 1996). The third domain is the cysteine-rich domain which consists of two EF hand-like motifs (Koenig et al., 1988), surrounded by WW (Bork and Sudol, 1994) and ZZ domains (Ponting et al., 1996) which are essential for binding to β-dystroglycan (Ishikawa-Sakurai et al., 2004). Finally, the carboxy-terminal domain consists of coil domains that are involved with protein binding (Koenig et al., 1988). Expression of the full-length dystrophin isoforms is controlled by three independent promoters; the brain (Dp427c), muscle (Dp427m) and Purkinje (Dp427p) that consist of unique first exons spliced to 78 common exons (Boyce et al., 1991, Chelly et al., 1990b, Gorecki et al., 1992). Dp427c is expressed mainly in cortical neurons and in the hippocampus (Barnea et al., 1990, Chelly et al., 1990b), whereas Dp427p is expressed in cerebellar Purkinje cells and in low levels in skeletal muscle (Gorecki et al., 1992, Holder et al., 1996). Dp427m is expressed in skeletal muscle and cardiomyocytes, with low levels also present in brain glial cells (Barnea et al., 1990, Chelly et al., 1990b). Additionally, there are four internal promoters that express truncated shorter dystrophin isoforms retaining the carboxy-terminus and are so named because of their molecular weight (Figure 1.2). Dp260 is expressed in the retina, with two isoforms derived from alternative splicing of the first exon (D'Souza et al., 1995). Dp140 is found throughout the central nervous system (Lidov et al., 1995) and has also been implicated in kidney development (Durbeej et al., 1997). Dp116 is expressed in Schwann cells (Byers et al., 1993), whilst Dp71 can be detected in most non-muscle tissues including brain, kidney, liver and lung (Blake et al., 1992, Hugnot et al., 1992, Lederfein et al., 1992). The number of expressed isoforms is increased further by variation derived from alternative splicing at the 3' end of the dystrophin gene (Bies et al., 1992, Feener et al., 1989). Although the cellular function of these shorter isoforms has yet to be fully determined, as they contain binding sites for dystrophin-associated proteins, they are thought to be involved in stabilisation and function of non-muscle dystrophin-like protein
Figure 1.2 Structure of dystrophin isoforms
Figure 1.2 Schematic structure of Dp427, Dp260, Dp140, Dp116 and Dp71 dystrophin isoforms and the dystrophin homologue, utrophin. Full-length dystrophin has four domains; the N-terminal actin binding domain (orange), the central rod domain with 24 spectrin-like repeats (blue oval) separated by 4 hinges (black), the cysteine rich domain (red) and the C-terminal domain (pale green). Also indicated are the positions of the 79 exons relative to the protein structure.

complexes (Blake et al., 2002). As Dp427c, Dp427p, DP140 and Dp116 are expressed in the brain and central nervous system, mutations that affect these isoforms may also account for the mental impairment observed in some DMD and BMD patients (Gorecki et al., 1992).

1.1.5 Homologues of dystrophin

As well as the various isoforms of dystrophin, homologues of the protein exist; namely utrophin, dystrophin related protein 2 (DRP2) and dystrobrevins. The gene for utrophin is located on chromosome 6 (Buckle et al., 1990) and encodes a 13Kb mRNA that produces a 395kDa protein (Tinsley et al., 1992) whose primary structure is similar to that of dystrophin (Figure 1.2). Urophin is widely expressed throughout the body including skeletal, cardiac (Pons et al., 1994) and smooth muscle (Nguyen et al., 1991) as well as vascular endothelia (Matsumura et al., 1993a) and Schwann cells of the peripheral nerves (Matsumura et al., 1993b), amongst other regions. In healthy adult muscle, utrophin is localised to the neuromuscular and myotendinous junctions (Nguyen et al., 1991, Ohlendieck et al., 1991), whereas in developing and regenerating muscle, it is distributed along the sarcolemma (Clerk et al., 1993, Gramolini et al., 1999b, Lanfossi et al., 1999, Lin et al., 1998, Takemitsu et al., 1991). Although utrophin is thought to have a role in maintenance of the post-synaptic membrane and acetylcholine receptor clustering (Nguyen et al., 1991), there is also evidence it can bind β-dystroglycan (Matsumura et al., 1992, Tommasi di Vignano et al., 2000), α-dystrobrevin (Peters et al., 1998) and syntrophins (Kramarcy et al., 1994, Peters et al., 1997). The similarities in structure and binding partners, as well as evidence of utrophin upregulation in DMD patients (Galvagni et al.,
2002), suggests a functional redundancy with dystrophin and as such, utrophin upregulation is being investigated as a potential therapy.

The DRP2 protein has a similar structure to Dp116, although it has a unique proline-rich N-terminal (Byers et al., 1993), and is expressed in the brain, associated with postsynaptic densities and cholinergic neurons (Roberts and Sheng, 2000). The dystrobrevin family of proteins bind directly to dystrophin and have significant protein sequence homology to the C-terminus of dystrophin (Wagner et al., 1993). Alpha-dystrobrevin has been proposed to play a role in intracellular signal transduction as well as interacting with utrophin at the neuromuscular junction (Blake et al., 2002). Beta-dystrobrevin is expressed in non-muscle tissues and complexes with Dp71 and utrophin (Blake et al., 1999, Loh et al., 2000) and is associated with various syntrophins (Kachinsky et al., 1999).

1.1.6 Role of dystrophin

Dystrophin is localised to the muscle sarcolemma, particularly within cytoskeletal lattices called costameres (Porter et al., 1992). Through interaction with a large protein network, costameres physically couple the sarcolemma with the Z-disc of force generating myofibrils (Tinsley et al., 1998). Dystrophin forms part of a large multimeric complex known as the dystrophin-associated glycoprotein complex (DGC). This complex is comprised of dystroglycan, sarcospan and sarcoglycans, which are all transmembrane proteins, and dystrobrevin and syntrophins (Figure 1.3). Dystrophin binds to β-dystroglycan, which in turn is bound to α-dystroglycan. This protein then interacts with the extracellular laminin-2, such that it provides a structural link between the cytoskeleton (through F-actin binding) and the extracellular matrix. The sarcoglycan complex is composed of α-, β-, γ- and δ- sarcoglycan. Although its function if not completely understood, this complex appears to strengthen the interaction of β-dystroglycan with α-dystroglycan and dystrophin (Ozawa et al., 2005). Within the cytoplasm, dystrophin binds to α-dystrobrevin, which interacts with the sarcoglycan complex (Yoshida et al., 2000) and binds α-syntrophin. Syntrophins are thought to function in anchoring ion channels and signalling molecules and α-syntrophin recruits the enzyme neuronal nitric oxide synthase
(nNOS) to the sarcolemma (Brenman et al., 1995). Alpha-dystrobrevin also binds syncoilin (Newey et al., 2001) and synemin (Mizuno et al., 2001), which interact with the intermediate filament protein network through binding with desmin. As well as abating any signalling function it may have, the absence of dystrophin results in the loss of the DGC complex, which in turn disrupts its role as a functional shock absorber to reduce mechanical strain that occurs during repeated rounds of contraction and relaxation.

![Diagram of the dystrophin glycoprotein complex](image)

**Figure 1.3 The dystrophin glycoprotein complex**

Dystrophin provides a structural link between cytoskeletal actin and the extracellular membrane, through interaction with laminin-2, via binding with α-dystroglycan. Dystrophin also has a role in signalling through binding of α-dystrobrevin and syntrophin, which recruits nNOS to the sarcolemma.
1.1.7 DMD pathophysiology

In patients with DMD, muscles characteristically have necrotic or degenerating muscle fibres, often in the presence of immature centrally nucleated fibres that represent muscle regeneration from myoblasts (Schmalbruch, 1984). As the regenerative capacity of the muscle decreases over time, muscle fibres are replaced by adipose and connective tissue. Absence of dystrophin results in disruption of the DGC that normally form costameres, lattice structures that anchor the cytoskeleton to the extracellular matrix (Rybakova et al., 2000). These structures distribute contractile forces that are generated in the sarcomere laterally though the sarcolemma to the basal lamina, maintaining a uniform sarcomere length along the fibre (Danowski et al., 1992). As a result of this disruption, the fibre membrane becomes more fragile, resulting in increased permeability, especially after sustained exercise (Hamer et al., 2002). This “leakiness” results in a high influx of extracellular calcium such that the fibre does not have the capacity to maintain physiological calcium levels. This sustained increase can lead to protease activation, particularly calpains, which results in further destruction of membrane proteins, perpetuating the calcium influx (Alderton and Steinhardt, 2000). Subsequent calcium overloading of the mitochondria may also induce down-regulation of mitochondrial gene transcription, eventually leading to metabolic crisis and necrosis (Chen et al., 2000). Loss of nNOS from the sarcolemma in DMD may also contribute to the DMD pathology. As nNOS synthesises the vasodilator, nitric oxide, its absence is thought to contribute to functional ischemia in muscle during exercise (Crosbie, 2001). Following muscle fibre necrosis, inflammatory cell infiltration, particularly of T-lymphocytes, can subsequently increase fibrosis, further contributing to the DMD phenotype (McDouall et al., 1990).

1.1.8 The DMD gene

The DMD gene spans approximately 2.5Mb of genomic sequence (Monaco et al., 1992) with the full-length mature 14-kb transcript (Koenig et al., 1987) composed of 79 exons (Roberts et al., 1993). Although genes with larger mRNAs exist, the DMD gene is the largest in the human genome, mainly due to the large introns that account for 99.4% of the gene (Ahn and Kunkel, 1993). It takes approximately 16 hours to transcribe the DMD
gene (Tennyson et al., 1995), which is a significant feat given that the splicing machinery must recognise exons that are separated by upwards of 250kb of intronic sequence. As well as transcribing full-length isoforms from varying tissue-specific promoters, dystrophin diversification occurs via alternative splicing throughout the coding region (Sironi et al., 2002). The majority of alternative spliced transcripts encoding the rod and cysteine-rich domains are in-frame (Sironi et al., 2002), which contrasts with the 5’ region of the dystrophin transcript where approximately only 50% of alternative transcripts maintain the reading frame (Surono et al., 1997). At the carboxy-terminus, the in-frame exon 71 and out-of-frame exon 78, are absent from approximately 50% of transcripts (Feener et al., 1989). The function of these alternative transcripts are unknown, but given that a large number are in-frame, and the most transcript variation is detected in the complex environment of the brain; it could be speculated that these transcripts do have a biological function and are not merely the result of splicing errors (Sironi et al., 2002).

1.1.9 Revertant Fibres

Alternative splicing has been proposed as the mechanism for the induction of dystrophin-positive “revertant” fibres, which occur at an incidence of 0-70% in muscles of DMD patients (Burrow et al., 1991, Fanin et al., 1995, Klein et al., 1992). These “revertant fibres” grow in a clonal fashion and although they do not occur at a high enough frequency to benefit DMD patients, the revertant dystrophin proteins appear to protect the muscle fibre from degeneration (Lu et al., 2000). There are two suggested mechanisms for the occurrence of these fibres; somatic reversion and alternative splicing. Somatic reversion, where a secondary mutation restores the mRNA reading frame, is consistent with the clonal growth and expansion of revertant clusters (Klein et al., 1992). However, as mutations are generally proliferation-related events, it could be expected that mutations would occur during prenatal muscle development, such that large clusters of “revertant fibres” would be present in neonates, which is not the case (Lu et al., 2000). Failure to detect loss of genomic sequences in the nuclei of “revertant fibres” by in situ hybridisation in mdx mouse tissue is also evidence against a secondary genomic deletion being the mechanism (Lu et al., 2000). An alternative splicing mechanism would be the simplest explanation for the variable exon removal that is reported in transcripts from mdx and normal mice (Lu et al.,
Given that "revertant fibres" in neonatal mice are single, rather in clusters, it would suggest that onset of the revertant phenotype corresponds to muscle differentiation, where regulation of alternative splicing is established (Lu et al., 2000). Although the clonal nature of "revertant fibre" expansion does not fit the alternative splicing model, it has been proposed that if an aberrant splicing pattern can produce a functional product, it may extend to other domains of the fibre and adjacent fibres undergoing degeneration as a form of "splicing imprinting", as it would confer a selective advantage (Lu et al., 2000). Regardless of the mechanism, there doesn’t appear to be any evidence that "revertant fibres" can improve the prognosis for DMD patients (Fanin et al., 1995, Nicholson et al., 1993); however their presence may act to tolerate the immune system to dystrophin, which is an important consideration for introduction of a functional dystrophin by gene therapy.

1.1.10 Mutations in the DMD gene

Due to its large size, the mutation rate of $1 \times 10^{-4}$ for the dystrophin gene is high compared to the $10^{-5}$ to $10^{-6}$ average for human genes (Nachman, 2004). There is a broad variety of mutations, with one third of all BMD and DMD mutations occurring as de novo events (Laing, 1993). In the Leiden DMD mutation database for example, over 2700 unique mutations have been reported (Fokkema et al., 2005). Of these, intragenic deletions of one or more exons are the most common, reported in ~65-70% of patients (Aartsma-Rus et al., 2006b, Koenig et al., 1989, Muntoni et al., 2003, White et al., 2002), while duplications account for ~6-8% of patients (Galvagni et al., 1994, White et al., 2002). The majority of these mutations are located in two regions, a major "hotspot" region which spans exons 45-53 and a minor "hotspot" region from exon 2-20 (Beggs et al., 1990, Liechti-Gallati et al., 1989). The most common deletions are exon 45 and exons 45-47, with the highest number of breakpoints located in introns 2, 7 and 44 (Aartsma-Rus et al., 2006b). The clustering of deletions simplifies diagnosis, with 98% of DMD/BMD deletions detectable by multiplex PCR of only 8 exons (Beggs et al., 1990). Approximately 20-30% of mutations involve small deletions, insertions or point mutations that result in induction of frame-shifts or nonsense codons (Aartsma-Rus et al., 2006b, Roberts et al.,
1994), with a significant number affecting splicing either by disrupting natural splice sites or activating cryptic splice sites.

1.1.11 The reading-frame hypothesis

The 'reading frame hypothesis' whereby in-frame deletions are found in BMD patients and out-of-frame deletions in DMD patients; holds true for over 90% of patients (Koenig et al., 1989). However the phenotype of these in-frame deletions is dependent on the location and size of the deletion (Arikawa-Hirasawa et al., 1995, Beggs et al., 1991, Fanin et al., 1996). Deletions in the central rod domain are generally less severe, with deletions of up to 35 exons reported with relatively mild BMD (England et al., 1990, Mirabella et al., 1998). However, in-frame deletions larger than this have a DMD phenotype, indicating that at least some part of the rod domain is necessary for function (Fanin et al., 1996). Deletions in the region encoding the actin-binding domain within the minor hotspot generally cause a severe BMD (Beggs et al., 1991), however a deletion of exons 3-9 was reported in an individual who was unaware of any symptoms until age 65 (Beggs et al., 1991). Although loss of the actin-binding domain would be expected to cause DMD, the presence of the central actin-binding domain may compensate to reduce severity (Rybakova et al., 1996). Further evidence for this is provided by the in-frame deletions removing the actin-binding domain and part of the central rod usually causing DMD (Arikawa-Hirasawa et al., 1995, Fanin et al., 1996, Vainzof et al., 1993). In-frame deletions in the cysteine-rich domain have not been described for BMD patients, indicating that this region is probably essential for function (Rafael et al., 1996), whilst in-frame deletions in the C-terminal also generally result in a DMD phenotype (Bies et al., 1992). As well as the location of the mutation, the amount of dystrophin protein can also affect severity. Generally, the more dystrophin protein produced, the milder the phenotype (Angelini et al., 1994), with DMD characterised by having none to 3% normal dystrophin levels and BMD characterised by greater than 20% dystrophin, although of abnormal size.

There are a number of exceptions to the reading frame rule with certain in-frame deletions associated with a DMD phenotype. In the Leiden database, in-frame deletions
and duplications are found in 7% of DMD patients, presumably because the in-frame deletion was too large or in a region critical for function (Aartsma-Rus et al., 2006b). For in-frame duplications, which are most commonly located in the actin-binding domain, the mutation disrupts the function of the domain (Aartsma-Rus et al., 2006b). There is also the possibility that deletions or duplications at the genomic DNA level can affect mRNA splicing such that out-of-frame transcripts are produced, as was the case for several DMD patients with an in-frame deletion of exon 5 (Gualandi et al., 2003). In a further exception to the reading frame rule, approximately 2% of patients with out-of-frame deletions and duplications at the genomic level, have a BMD phenotype (Aartsma-Rus et al., 2006b). Similarly, nonsense mutations do occur in BMD patients. Two different nonsense mutations in exon 29 were found to disrupt exon splicing enhancer motifs such that the in-frame exon, and hence the truncating mutation, were omitted during splicing, permitting production of significant levels of dystrophin lacking exon 29 (Ginjaar et al., 2000).

Further complicating the correlation of mutation to phenotype is genomic deletions that are found in both DMD and BMD patients. The most common of these is a deletion of exons 3-7 and although alternative splicing of exon 2 or 8/9 could restore the reading frame, and has been detected at low levels in some BMD patients (Chelly et al., 1990a), this does not occur in the majority of these patients (Gangopadhyay et al., 1992, Winnard et al., 1993, Winnard et al., 1995). Another proposed mechanism is the usage of an alternative translation initiation codon located in exon 8, resulting in some BMD patient transcripts where exon 8 but not exon 1 or 2 was present (Gangopadhyay et al., 1992, Winnard et al., 1993, Winnard et al., 1995). Thus, although the majority of dystrophin mutations adhere to the reading-frame rule, there are exceptions to this at the genomic DNA level, thus confirmation of mutation at the RNA level should be used if possible (Aartsma-Rus et al., 2006b).
1.2.1 Mouse models

A number of animal models have been reported with a spontaneous mutation in the dystrophin gene that has resulted in their utilisation for studies of potential therapies. In the mdx mouse model, a point mutation in exon 23 induces a termination codon (Sicinski et al., 1989) such that a truncated non-functional protein is translated (Hoffman et al., 1987). Despite lacking the full-length dystrophin isoform, there is no obvious weakness and they have a near-normal lifespan (Pastoret and Sebille, 1995). A muscle pathology does exist though; with hypertrophy, necrosis (especially at 3-4 weeks of age) (Tanabe et al., 1986) and continuous cycles of fibre regeneration and degeneration characterised by centrally nucleated fibres (DiMario et al., 1991, Lynch et al., 2001). There is marked fibre loss and fibrosis, particularly in the diaphragm (Stedman et al., 1991) which increases significantly in the limb muscles of older mdx mice (Pastoret and Sebille, 1993). Functionally there is a reduction in normalised force production and power output (Lynch et al., 2001), however there is not the severe pathophysiology that is associated with the DMD patient phenotype. The absence of dystrophin though does make this animal model useful for testing of potential therapies, and it has been the most widely employed in studies to date. A number of mouse models have also been generated by ethyl-nitroso-urea mediated mutagenesis, which induces point mutations (Chapman et al., 1989). Like the mdx model, these mdx<sup>2cv</sup>-<sup>scv</sup> mice display only a mild disease phenotype, although the mdx<sup>3cv</sup> is interesting in that the dystrophin mutation lies proximal to the Dp71 promoter, such that all of the dystrophin isoforms are absent (Cox et al., 1993b).

Transgenic mouse models have also been developed including a dystrophin and utrophin null mouse that more closely resembles the DMD phenotype, with a severe progressive muscle dystrophy and death before 20 weeks (Deconinck et al., 1997, Grady et al., 1997). Although not a true genetic model of DMD, as patients have a functional utrophin gene, it still has potential for evaluation of different therapies, especially in the
understanding of the central nervous system where both dystrophin and utrophin are normally expressed. Other mouse models based on the \textit{mdx} mouse have been developed to contain partial dystrophin cDNAs transgenes, so as to identify regions of the gene essential for function (Cox et al., 1993a, Cox et al., 1994, Greenberg et al., 1994, Rafael et al., 1996). These experiments demonstrated that internally deleted transgenes, missing even 46\% of the coding region, yet containing both the N- and C-terminal domains, could prevent a dystrophic pathology (Phelps et al., 1995, Wells et al., 1995). Expression of the Dp71 isoform though, whilst able to localise the DGC, was unable to prevent the \textit{mdx} pathology (Cox et al., 1994, Greenberg et al., 1994, Phelps et al., 1995, Wells et al., 1995).

Finally, a transgenic mouse model was recently developed to express the entire human DMD gene on a yeast artificial chromosome, with yeast spheroblasts fused to mouse embryonic stem cells to generate mice homozygous for the human DMD gene (hDMD) (Bremmer-Bout et al., 2004). When crossed with the \textit{mdx} mouse, the presence of human dystrophin was able to abrogate the dystrophic phenotype and restore the sarcolemmal localisation of \(\alpha\)-sarcoglycan and \(\beta\)-dystroglycan (Bremmer-Bout et al., 2004). It is proposed that this model could be used to optimize target sequence, dosage and administration of human specific AOs prior to application to DMD patients. However, the relevance of an AO treatment optimized in a mouse splicing background remains questionable.

\subsection*{1.2.2 GRMD model}

A number of dystrophin-deficient canine models have been identified, with the best characterized of these identified in 1988 when a spontaneously occurring muscular dystrophy was described in a litter of golden retriever dogs (Kornegay et al., 1988) and a colony established. These dogs, termed the \textit{Golden Retriever Muscular Dystrophy} (GRMD) model, have low levels of dystrophin transcripts (undetectable by Northern blot analysis (Sharp et al., 1992)) and absence of dystrophin protein (Cooper et al., 1988). They have striking phenotypic similarities to DMD, including elevated serum creatine kinase levels, and weakness and stiffness of gait by about 6 to 8 weeks (Valentine et al., 1992).
There is a progressive stiffness of the limb muscles, with overextension of the carpus, overflexion of the tarps and abduction of the paws (Kornegay et al., 1994a, Kornegay et al., 1994b). Myopathic changes show muscle atrophy with contractures, myofibre degeneration with mineralization, fibrosis with fatty infiltration and cardiomyopathy (Nguyen et al., 2002). Affected animals can live for a few months to a couple of years, with mature animals usually dying from cardiac dysfunction (Valentine et al., 1989). Considerable variation in severity is occasionally observed in the same litter with animals termed a neonatal fulminating form dying within a few days of birth, with pale streaking in muscles caused by necrosis and calcification observed (Howell et al., 1997).

The genetic basis for the dystrophin deficiency is a point mutation in the 3' consensus splice site of intron 6 that disrupts the splice site such that splicing machinery fails to recognize exon 7, precluding it from the mature dystrophin mRNA (Sharp et al., 1992). Loss of this exon induces a frame-shift in the mRNA reading frame with a premature termination codon introduced within exon 8 (Sharp et al., 1992). Extensive studies undertaken in the mdx mouse model have not been replicated in the GRMD model due to its relative scarcity. Only a few colonies have been established, presumably due to the high cost and technical expertise necessary to maintain them. Additionally, the longer gestation period and variability of affected litter numbers also contribute to the low numbers of animals available for study.

1.2.3 Other DMD animal models

A deletion of the Dp427m and Dp427p promoter regions of the dystrophin gene is the cause of the pathological features of the hypertrophic feline muscular dystrophy (Gaschen et al., 1992, Winand et al., 1994). Although these animals have an abnormal gait and low levels of necrotic fibres, fibrosis is not observed and there is marked fibre hypertrophy. Although studies have been performed to characterize this model it has yet to be employed for in vivo testing of potential therapies.
Recently, zebrafish have emerged as potential disease models for genetic disorders. This animal has a number of traits that make it attractive for study including its small size, rapid ex-utero development, high reproductive capacity and short generation time (Guyon et al., 2007). Zebrafish genomes also have strong similarity to the human genomes (Woods et al., 2000) with orthologous genes demonstrated to regulate similar developmental processes (Langenau and Zon, 2005). However, as zebrafish are evolutionarily more distant from humans than mammalian models, it is likely that any conclusions from fish experiments would need to be replicated in mammals before application to humans. In a screen of mutant zebrafish families, a subgroup of fish that developed normally then underwent muscle degeneration, were identified to have a nonsense mutation in exon 4 of the dystrophin gene (Bassett et al., 2003). Evidence for similarities in biochemistry of human and zebrafish DPGs was provided by experiments targeting dystrophin in developing embryos with short interfering RNA resulting in disorganized sarcomeres and body defects (Dodd et al., 2004). Similarly, inhibition of dystrophin translation by morpholinos caused a loss in zebrafish activity (Guyon et al., 2003). Zebrafish have the potential to evaluate a number of different therapeutic approaches for DMD. They are particularly an ideal model for drug screening in a living organism, as chemicals could be introduced to their water environment such that any that reduced the symptoms of the dystrophic mutant could be considered for further evaluation (Guyon et al., 2007). Zebrafish could also be useful for gene upregulation studies as transgenic fish are much easier to create than mice. Alternately, direct injection of cDNA would be a simple method for transient protein expression.
1.3 THERAPIES FOR DMD

1.3.1 Preventative care

It has been twenty years since the DMD gene was identified, yet there is still no treatment for DMD that addresses the genetic defect. Whilst a number of genetic therapeutic approaches are being developed, current treatment strategies focus on supportive and preventative care as well as corticosteroid administration. The development of joint contractures severely affects the quality of life in boys prior to loss of ambulation. Whilst studies have shown that passive stretching exercises do not show a benefit, the use of leg braces and heel splints at night were correlated to reduced contractures (Brooke et al., 1989). Exercise in ambulant DMD patients has also been demonstrated to have benefit, especially sub-maximal exercise (de Lateur and Giaconi, 1979) and during the first few months of training (Ansved, 2003). With the loss of ambulation, scoliosis becomes a common orthopedic problem in DMD patients. Scoliosis can exaggerate diaphragmatic weakness, thus reducing lung vital capacity which is already compromised (Strober, 2006). As surgery becomes riskier over time due to cardiopulmonary weaknesses, spinal correction is commonly performed before vital capacity is below 30-35% (Schramm, 2000). Besides diaphragm weakness, thoracic and abdominal muscle weakness can lead to restrictive lung disease such that nocturnal hypoventilation and hypoxemia can occur. This can be treated with noninvasive positive pressure ventilation that can improve survival (Bach et al., 1997), mental health and social function (Simonds et al., 1998).

1.3.2 Corticosteroids

The benefits of corticosteroid use in DMD was first demonstrated in a 1974 study of 14 boys where prednisone treatment lead to stabilization or improvement of functional status (Drachman et al., 1974). The first randomized, double-blind placebo controlled study showed improvement of strength testing in the first three months, followed by natural progression similar to control groups (Mendell et al., 1989). Other large studies have
reported that daily low dosing (0.75mg/kg) was preferable to larger dosage (2.5mg/kg) on alternate days (Fenichel et al., 1991). Various dosing regimens using overall lower doses, such as 10 days on, 10 days off, have been tested in small patient studies in an attempt to reduce the significant side effects associated with prednisone, including cushingoid features and weight gain (Kinali et al., 2002). An alternative steroid in use is Deflazacort, an oxazoline derivative of prednisone. In a randomized, double-blind, placebo controlled study, administration of 2mg/kg on alternate days was found to slow progression of weakness compared to control groups, with less severe side effects than prednisone (Manzur et al., 2004). However, approximately one-third of boys develop asymptomatic cataracts (Biggar et al., 2001, Biggar et al., 2004), which is rarely reported in prednisone treatment, and the drug has not been approved for use in the USA. A comparative study of prednisone and Deflazacort treatment reported no statistically significant differences between the two treatment groups for either strength or function (Bonifati et al., 2000). A review of published large scale studies by the Quality Standards Subcommittee of the American Academy of Neurology led to a published recommendation of 0.75mg/kg/day of prednisone as a treatment, with gradual reduction to 0.3mg/kg/day if side effects occur, although they also noted that Deflazacort could be used in countries where it was available (Moxley et al., 2005).

The exact mechanism of action of corticosteroids is unknown although they do have anti-inflammatory and immunosuppressive properties. Muscle biopsies from prednisone treated patients exhibited a reduction in the number of cytotoxic/suppressor T cells, which in turn could lead to a decrease in cytokine activity (Kissel et al., 1991). However, suppression of the inflammation process alone is insufficient for clinical improvement, as trials with the immunosuppressant azathioprine demonstrated (Kissel et al., 1993). Corticosteroids also up-regulate skeletal muscle genes, including those important in muscle hypertrophy (Fisher et al., 2005), as well as promote proliferation and/or fusion of muscle precursor cells in mdx mice in response to injury (Anderson et al., 1996).
1.3.3 Other pharmaceuticals

Whilst glucocorticoids are thought to act on a number of levels to reduce the dystrophic pathology in DMD, other pharmaceuticals could potentially be employed to treat specific secondary effects. Pathological changes caused by calcium accumulation could be addressed by targeting of ion channels. Drugs such as pentoxifylline have been demonstrated to block exercise-sensitive calcium channels (Rolland et al., 2006) whilst antibiotics that act to block stretch activated channels reduce muscle fibre necrosis in mdx mice and creatine kinase levels in DMD patients (Whitehead et al., 2006). Pentoxifylline also has a wide range of anti-inflammatory effects, reducing TNFα production in vitro (Vary et al., 1999) and increasing muscle strength in exercised adult mdx mice (Granchelli et al., 2000). There is evidence that aggressive inflammation in DMD contributes to muscle necrosis, thus immunosuppressive drugs such as cyclosporine (Sharma et al., 1993) and more recently pentoxifylline, have been the subject of clinical trials in DMD patients.

As well as broadly acting anti-inflammatory drugs, the ability to modulate specific cytokines has been shown in severe inflammatory disorders such as Crohn’s disease (Feldmann and Maini, 2003). TNF-α is a key pro-inflammatory cytokine and blockage of activity with the neutralizing antibody infliximab, has been shown to delay and reduce muscle necrosis in young mdx mice (Grounds and Torrisi, 2004). Use of soluble receptors to TNF-α also inhibits activity such that it reduces muscle necrosis in young mdx mice (Pierno et al., 2006) and exercised adult mice (Hodgetts et al., 2006) with additional benefits to muscle strength and reduction in creatine kinase levels.

Antioxidants can reduce the levels of reactive oxygen species that have the potential to damage muscle tissue. Supplementation of green tea extracts to mdx mice diets showed a reduction in muscle damage and improved muscle function in two separate studies (Buetler et al., 2002, Dorchies et al., 2006). Coenzyme Q10 has also been used in a pilot DMD clinical study, in combination with steroid treatment, where increased strength in some muscle groups was observed, suggestive of a beneficial effect that is currently being assessed in a larger scale clinical trial (http://www.cinrgresearch.org/). Calpains, which are
calcium activated proteases, are increased in dystrophic muscle and have been implicated in myofibre degeneration (Spencer et al., 1995). The calpain inhibitor, leupeptin, reduced levels of muscle necrosis in \textit{mdx} mice (Badalamente and Stracher, 2000) whilst a new compound BN 82270, has dual action as a calpain inhibitor and antioxidant, and demonstrated increased muscle strength, decreased serum CK and reduced fibrosis of the \textit{mdx} diaphragm following treatment (Burdi et al., 2006).

Several nutritional supplements have been evaluated for their ability to increase muscle strength and mass in DMD patients. Creatine monohydrate, an amino acid that stores energy as phosphocreatine in the muscle, was demonstrated to increase hand-grip strength and fat-free mass in a four month trial of DMD patients (Tarnopolsky et al., 2004). A more recent clinical trial concluded that although creatine did not significantly increase muscle strength, it was well tolerated and there appeared to be a trend towards less deterioration (Escolar et al., 2005). The reduction of nitric oxide levels observed in DMD can be potentially addressed by the administration of L-arginine, which has been demonstrated to enhance nitric oxide synthesis as well as up-regulate expression of utrophin (Chaubourt et al., 2002). Systemic supplementation of L-arginine was shown to decrease muscle necrosis and dystrophic pathology in \textit{mdx} mice (Voisin et al., 2005) and when given in combination with Deflazacort, reduced exercise induced muscle damage (Archer et al., 2006).

The importance of pharmaceutical or nutritional therapeutics in the treatment of DMD should not be underestimated. Whilst genetic therapies are being developed to address the primary genetic defect, pharmaceutical intervention, particularly corticosteroids, can reduce or at least help stabilize the progressive weakness. Further investigation of potential drugs that target multiple facets of the dystrophic pathology will be necessary, but it is not unrealistic to expect that the degenerative process can be at least delayed and skeletal muscle mass and function improved.
1.3.4 Myoblast transfer

In skeletal muscle, multi-nucleated fibres are formed by the fusion of mononuclear cells called myoblasts during embryogenesis. Adjacent to these mature muscle fibres is a population of “satellite cells” which are quiescent, mononucleated myogenic stem cells (Campion, 1984) that have the ability to proliferate and fuse to repair or replace damaged myofibres (Carlson and Faulkner, 1983). Continual cycles of muscle degeneration and regeneration in DMD depletes the satellite cell population such that they are no longer able to compensate for muscle damage. It has been proposed then that the introduction of myoblast precursor cells could participate in regeneration of muscle fibres and was subsequently first demonstrated in 1979 (Lipton and Schultz, 1979). Transplantation of wild-type derived myoblasts into mdx muscle resulted in fusion with recipient fibres and subsequently high levels of dystrophin expressing fibres (Morgan et al., 1990, Partridge et al., 1989). Based on this success, a number of clinical trials were undertaken with DMD patients (Gussoni et al., 1992, Huard et al., 1992, Karpati et al., 1993, Mendell et al., 1995, Miller et al., 1997, Tremblay et al., 1993). These trials were largely unsuccessful, with only a small increase in dystrophin-positive fibres observed in transplanted muscles (Huard et al., 1992, Mendell et al., 1995, Miller et al., 1997), and functional testing demonstrating at best, a transient strength increase in a few patients from one of the clinical trials (Huard et al., 1992). Large scale clinical trials of myoblast transfer were performed that reported some functional improvement and presence of dystrophin in transplanted muscles (Law et al., 1993, Law et al., 1997), however these dystrophin-positive fibres were later identified to be ‘revertant fibres’ rather than derived from donor myoblasts (Partridge et al., 1998). This failure was mainly due to immunological rejection of donor myoblasts (Gill and Wolf, 1995), that can cause up to 95% loss of donor myoblasts after two days (Qu et al., 1998). Myoblast survival can be improved through the use of immunosuppressive drugs such as FK506 or cyclosporine (Smythe et al., 2000), although they have severe adverse effects in clinical protocols including nephro- (Klintmalm and Gonwa, 1995) and neurotoxicity (Neu et al., 1997). More recently, clinical trials were performed with trial design based on non-human primate experiments (Kinoshita et al., 1995, Skuk et al., 2000). Myoblasts derived from healthy fathers, were transplanted using a high-density intramuscular injection protocol in a 1cm$^3$ region, in the presence of the immunosuppressant drug, tacrolimus (Skuk et al., 2006, Skuk et al., 2004). In eight patients, 3% to 26% of the profiled
myofibres were found to express donor-derived dystrophin. This “high-density” injection protocol was extended to whole muscles in a clinical trial of only a single DMD patient (Skuk et al., 2007). Repetitive injections of myoblasts derived from the father were administered such that 100-200 injections per cm\(^2\) were applied over the surface of the muscle, with approximately 2,100 injections performed in the biceps brachii, amongst other muscles (Skuk et al., 2007). Myoblast transfer success was variable with approximately 35% of myofibres expressing dystrophin in treated gastrocnemius muscle, 18 months post-treatment. However, almost no dystrophin-positive myofibres were present in the biceps, where the skeletal muscle parenchyma was virtually absent, indicating that innervated myofibres may need to be present to provide the scaffold to incorporate transplanted myoblasts (Skuk et al., 2007). Whilst normal myoblast allotransplantation with a high-density injection protocol was feasible, it is debatable if this procedure could be performed in a clinical setting to address all muscles, especially muscles that are not readily accessible from the skin surface.

### 1.3.5 Other stem cell therapies

Due to the limited success of myoblast transfer to date, other stem cells have been proposed as an alternative source of donor cells. Bone marrow-derived cells have been shown to participate in muscle formation (Ferrari et al., 1998), however transplantation into \textit{mdx} mice, even after enrichment of stem cells with myogenic potential, had a very poor outcome with no more than 2% expression of donor derived dystrophin-positive fibres (Fukada et al., 2002). Blood vessel-derived stem cells called mesoangioblasts, have been isolated from embryonic dorsal aorta and shown to form muscle and repopulate diseased muscle in mice lacking α-sarcoglycan (Galvez et al., 2006). The potential of these stem cells were recently investigated in the GRMD model, with mesoangioblasts extracted from vessels of young dogs injected systemically into the femoral artery (Sampaolesi et al., 2006). These cells were either normal heterologous cells delivered with immunosuppression, or autologous cells that had been genetically corrected. Although dystrophin-positive fibres were present in a number of animals, the level of dystrophin correction did not necessarily correlate well with functional improvement. For example, the healthiest animal had similar numbers of dystrophin-positive fibres in the injected and
non-injected contralateral muscles, whereas another animal with increased dystrophin-positive fibres in all muscles investigated had a major decline in health (Sampaolesi et al., 2006). In a critical review of this study it is suggested that the immunosuppression regimen may have had some benefit and could explain the somewhat contradictory results (Grounds and Davies, 2007), and thus further investigation is needed to determine if mesoangioblast transfer could have a benefit for DMD.

1.3.6 Gene replacement

Gene transfer to introduce of a functional copy of a gene has a number of inherent difficulties including producing sufficient quantities of delivery vector, cellular tropism of the vector, ectopic gene expression, immunological challenges and the need to achieve whole-body delivery (Odom et al., 2007). Compounding these challenges for DMD is the large size of the dystrophin cDNA that precludes its full-length expression in some viral vectors. It is known however, that a large range of deletions in dystrophin only cause a mild phenotype in BMD patients, indicating that not all regions are crucial for function. To map these regions, transgenic \textit{mdx} mice were engineered with various deletions throughout the four dystrophin domains (Figure 1.4). These studies indicated that a significant proportion of the rod domain can be lost whilst still retaining function. A 6.2kb mini-dystrophin construct that was designed to mimic a mild BMD exon 17-48 deletion (England et al., 1990), produced \textit{mdx} mice that had almost normal muscle morphology and force generation (Phelps et al., 1995). This construct though is still too large for recombinant adeno-associated viral (rAAV) vectors which have a packaging capacity of 4.9kb (Dong et al., 1996) and so an even smaller 3.6 kb micro-dystrophin construct was created that could prevent, and partially reverse, the dystrophic phenotype in \textit{mdx} mice when delivered to the muscle (Harper et al., 2002).
Figure 1.4 cDNA constructs for dystrophin gene replacement

Removal of the 5’ and 3’ UTRs from the 14kb dystrophin cDNA induces an 11kb full-length construct (Dp427). The mini-dystrophin construct is based on a mildly affected BMD patient with an exon 17-48 deletion that removes repeats 4-18 and the second hinge region. The micro-dystrophin construct is derived from functional deletion studies, with deletion of repeats 4-21, as well as hinge 3, the most commonly used in AAV studies. Both of the smaller constructs lack the C-terminal region (pale green) as they are functionally similar to constructs that retain this region (Crawford et al., 2000).

1.3.6.1 Adenoviral vectors

First generation adenoviruses were the first viral vectors to deliver a human mini-gene to the mdx mice by intramuscular injection, with approximately 50% of fibres expressing dystrophin for up to 3 months (Ragot et al., 1993). Whilst these early studies demonstrated good expression in neonatal mice, there was poorer transduction of adult muscle, with expression lost after 60 days (Acsadi et al., 1996, Ragot et al., 1993, Vincent et al., 1993, Zhao et al., 1997). This was proposed to be due to a combination of the reduced expression of the coxsackie/adenovirus receptor, which is involved in viral transduction (Feero et al., 1997, Nalbantoglu et al., 1999), and the maturation of the immune system. Indeed, improvement in dystrophin expression was demonstrated with the use of immunosuppressive methods that depleted the immune system (Guibinga et al., 1998, Petrof et al., 1996). Another issue that complicates clinical use of these vectors is that a large proportion of the population has pre-existing neutralizing antibodies to
adenovirus (Chirmule et al., 1999). To reduce these adverse effects, “gutted” adenoviral vectors were developed which lacked viral genes such that these vectors were less immunogenic (Clemens et al., 1996, Kochanek et al., 1996, Kumar-Singh and Chamberlain, 1996). Use of these vectors resulted in significant improvement in the duration of transgene expression (Chen et al., 1999, Morsy et al., 1998, Schiedner et al., 1998) although significantly, even gutted adenoviral vectors have been reported to induce an immune response (DelloRusso et al., 2002). Use of these “gutted” vectors has been used to express full-length dystrophin cDNA in mdx mice and GRMD animals, although gene expression was reduced compared to second generation adenovirus vectors (Gilbert et al., 2001) and it may be necessary to further optimize these vectors to include sufficient viral gene products for efficient expression whilst minimizing the immune response.

1.3.6.2 Adeno-associated viral vectors

With the limitations associated with adenoviral vectors, the potential of recombinant adeno-associated viral (rAAV) vectors for dystrophin gene transfer has been explored. rAAV vectors are deleted of all viral genes, requiring helper plasmids that encode rep and cap genes, the non-structural and structural viral proteins respectively. Their non-pathogenic nature, availability of serotypes with muscle tropism, relatively low immunogenicity and long-term transgene expression makes them an attractive candidate for DMD gene therapy (Odom et al., 2007). For the transduction of muscle cells, serotypes AAV-5, 6 and 8 have demonstrated enhanced transduction efficiency compared to AAV-2 (Blankinship et al., 2004, Chao et al., 2000, Duan et al., 2001). The major limitation of AAV use is that the expression cassette cannot exceed the wildtype genome size of 4.7kb (Grieger and Samulski, 2005). This precludes expression of the entire dystrophin cDNA, however the 3.6kb micro-dystrophin construct has been used successfully to ameliorate the dystrophic pathology following AAV-mediated delivery to the mdx muscle (Gregorevic et al., 2004, Liu et al., 2005, Roberts et al., 2002, Wang et al., 2000, Watchko et al., 2002, Yoshimura et al., 2004). AAV delivery was particularly effective when delivered to neonatal and young mice with fewer centrally nucleated fibres, increased force and increased resistance to contraction induced injury when compared to treated adult mdx mice (Liu et al., 2005, Yoshimura et al., 2004). To improve delivery of AAV vector, intravenous
injection without surgery or anesthesia, was used to transduce >90% of adult mouse musculature, including significantly the diaphragm and the heart, that was further improved by the inclusion of vascular endothelial growth factor (Gregorevic et al., 2004). Whilst micro-dystrophin expression can reduce the dystrophic phenotype, larger mini-dystrophin constructs may have improved functionality, although there is little data comparing these constructs (Blankinship et al., 2006). An alternative AAV strategy to allow delivery of mini-dystrophin has been to split the dystrophin coding sequence between two vectors, such that one contains the splice donor of a dystrophin intron, and the other the splice acceptor site for the same intron (Lai et al., 2005). This strategy could permit inclusion of a larger and more powerful gene cassette that would otherwise be unable to be expressed in a single rAAV vector.

One of the apparent advantages of AAV use is the low immunogenicity of AAV vectors that permits the long-term expression of the transgene. Most AAV studies though have been undertaken in mice, which do not always successfully predict the human clinical response. To determine if rAAV could be successfully delivered to more complex mammals, AAV-2 and AAV-6 vectors containing a β-galactosidase transgene, were delivered intramuscularly to random-bred normal dogs (Wang et al., 2007a). In contrast to successful mice studies, delivery to canine muscle induced a strong T-cell mediated immune response to viral proteins that was sufficient to largely eliminate transgene expression. The authors postulated that the lack of T-cell response in mice may reflect the naïveté of the murine immune system, compared to the more evolved immune surveillance in larger animals (Wang et al., 2007a). To address this limitation, a transient, but aggressive, course of immunosuppression was administered concurrently with intramuscular injection of an AAV micro-dystrophin cassette into a canine model of DMD (Wang et al., 2007b). Immune responses to both vector and transgene were suppressed during the 16-week course of immunosuppression with expression of canine micro-dystrophin persisting three months after withdrawal of immunosuppression (Wang et al., 2007b). Thus, whilst there is significant potential for AAV-mediated gene therapy, it remains to be determined if issues of immunogenicity may prohibit or limit its use in the clinical setting.
1.3.6.3 Non-viral vectors

One of the major complications in replacing the defective dystrophin gene is the large size of the coding sequence (~11Kb) that precludes the use of conventional viral vectors. An alternative has been the use of plasmid vectors, which have larger insert capacities and do not induce an immune response, unlike viral proteins. Delivery of plasmid vectors containing either full-length or mini-dystrophin cDNAs by direct injection to the muscle of either mdx mice (Acsadi et al., 1991, Decrouy et al., 1998, Ferrer et al., 2000) or GRMD animals (Zhao et al., 1997), resulted in low levels of dystrophin expression confined to the site of injection, which declined over time. Improved vector uptake, through the use of electroporation with hyaluronidase pretreatment, could enhance dystrophin expression such that the efficiency was similar to local delivery by viral vectors (McMahon et al., 2001). Delivery of full-length plasmid via tail vein injection, with short occlusion of the inferior vena cava, resulted in dystrophin expression in 40% of diaphragm fibres (Liu et al., 2001). Intravascular delivery of plasmid DNA into limb arteries of rats and non-human primates has been used to express high levels of foreign transgene with minimal muscle damage (Zhang et al., 2001). Application to the mdx model via intra-arterial delivery of naked full-length dystrophin cDNA, induced expression throughout the hind limb muscles, although notably this was only 1-5% of total muscle fibres (Zhang et al., 2004). A Phase I clinical trial of intramuscular injection of a full-length human dystrophin plasmid was performed in nine patients with DMD or BMD (Romero et al., 2004). Following a single or multiple injections into the radialis muscle, dystrophin could be detected in only six of the nine patients despite vector being detected at the injection site of all patients. This expression was low, with up to 6% complete sarcolemmal staining and up to 26% partial labeling, although significantly there were no adverse effects or cellular or humoral anti-dystrophin responses (Romero et al., 2004). As the first report of gene transfer into diseased human skeletal muscle, the apparent safe outcome was important, although an increase in dystrophin protein levels would be necessary to have a clinical benefit. Notably the authors are completing monkey and GRMD studies of larger doses via loco-regional intravascular delivery, with a view of improving plasmid uptake in future clinical trial studies.
1.3.7 Utrophin gene upregulation

An alternative approach to DMD therapy has been to use drug-based upregulation of utrophin expression to compensate for dystrophin dysfunction. The observation that utrophin is upregulated in *mdx* mice and DMD patients (Galvagni et al., 2002, Mizuno et al., 1993) led to the hypothesis that utrophin could potentially have a protective, as well as complementary role, in dystrophic muscle. This theory is further supported by utrophin-dystrophin double-deficient transgenic mice, which have a severe progressive phenotype and die prematurely (Deconinck et al., 1997, Grady et al., 1997). Although utrophin upregulation cannot be expected to fully compensate for the loss of dystrophin, adenoviral delivery of mini-utrophin was able to rescue the dystrophic phenotypes in *mdx, mdx-utr-* and canine models of DMD (Cerletti et al., 2003, Gilbert et al., 1999, Wakefield et al., 2000). Similarly, utrophin over-expression in transgenic *mdx* mice prevented the dystrophic phenotype entirely (Tinsley et al., 1998).

There are two full-length isoforms of utrophin with unique N-terminal regions: utrophin A, which is found in skeletal muscle fibres, and utrophin B which is located in vascular endothelium (Chakkalakal et al., 2003, Weir et al., 2002). As only utrophin A has been detected at the sarcolemma of DMD patients and *mdx* mice (Weir et al., 2002), the focus has been to upregulate this isoform. The utrophin promoter A contains binding sites for transcription factors Sp1, Sp3 and Ap2 which drive constitutive transcription (Perkins et al., 2001), an E-box that binds myogenic factors to regulate utrophin in a muscle specific manner (Funk et al., 1991), and a N-box motif that is responsible for synapse-specific utrophin expression (Koike et al., 1995). The nerve growth factor heregulin is known to stimulate N-box dependant utrophin transcription (Gramolini et al., 1999a) and administration of a small peptide region of heregulin was shown to increase utrophin levels threefold, ameliorating the dystrophic phenotype in *mdx* mice (Krag et al., 2004). Other compounds that increase utrophin expression through the N-box motif, such as nitric oxide (Wehling et al., 2001), L-arginine (Barton et al., 2005) and other modulators of nNOS activity like molsidomine (Voisin et al., 2005), have also improved the *mdx* mouse phenotype. Interestingly, the corticosteroid deflazacort, which is used as a treatment for some DMD patients, has also been shown to increase utrophin A expression when
administered to mdx mice (St-Pierre et al., 2004), suggestive that utrophin upregulation could be one of the mechanisms for its beneficial effects (Miura and Jasmin, 2006). To identify compounds that upregulate utrophin expression through unknown pathways, a reporter construct containing the utrophin A promoter region linked to a luciferase gene, has been generated to allow high-throughput drug screening of thousands of chemical compounds (Khurana and Davies, 2003). Although several candidate molecules have been identified in vitro to increase utrophin expression two to three-fold, they have yet to be verified in vivo (Miura and Jasmin, 2006), which will be especially important in the more severely affected GRMD model. There is also the issue of whether utrophin upregulation would be beneficial in older patients, as studies of mdx mice with an inducible utrophin transgene demonstrated that although the dystrophic phenotype could be prevented if expression was turned on in newborn mice, if delayed until they were 30 days old, improvement was only marginal (Squire et al., 2002).

1.3.8 Premature termination read-through

Nonsense mutations give rise to in-frame codons that lead to premature termination of translation such that truncated protein products are produced (Mendell and Dietz, 2001). In DMD, approximately 15% of patients carry a nonsense mutation that precludes translation of a functional dystrophin protein (Aartsma-Rus et al., 2006b). High concentrations of aminoglycosides, such as gentamicin, are known to promote read-through of these premature nonsense codons and studies in mdx mice demonstrated success with 10-20% of wild-type dystrophin levels observed following treatment (Barton-Davis et al., 1999). Clinical trials with gentamicin in BMD and DMD patients were less successful (Wagner et al., 2001), and subsequent mdx studies did not reproduce the encouraging initial results (Dunant et al., 2003). These contrasting results were speculated to be due to variation in batches of gentamicin, as multiple isoforms exist with both varying potency and toxicity. To identify other compounds that would suppress UGA nonsense codons, two high-throughput screens comprising approximately 800,000 low molecular weight compounds were performed (Welch et al., 2007). Compounds that demonstrated UGA read-through with minimal toxicity were characterized and PTC124 was identified as a potential candidate (Welch et al., 2007). PTC124 could suppress nonsense alleles in 30
muscle cell cultures from DMD patients and *mdx* mice, facilitating the production of dystrophin. These results were confirmed by oral, intraperitoneal or combined dosing of *mdx* mice for 2-8 weeks that resulted in near full-length dystrophin protein production in all skeletal muscles examined, and in cardiac muscle. Furthermore, partial functional recovery was also observed through improvement of specific force measurements in treated mice (Welch et al., 2007). Based on these positive findings, clinical trials have been initiated with PTC 124 and if successful, should be of great benefit to those DMD patients affected by nonsense mutations.

**1.4 ANTISENSE-MEDIATED EXON SKIPPING**

**1.4.1 Modification of splicing**

Antisense oligonucleotides (AOs) are short nucleic acids that can alter gene expression through a variety of mechanisms, including specific RNase H mediated degradation of the transcript (Zamecnik and Stephenson, 1978) and blockade of translation initiation (Chiang et al., 1991). Whilst these down-regulation approaches are potentially useful for dominant gene disorders, they are not applicable in those diseases caused by the loss of a functional protein such as DMD. An approach gaining considerable interest as a potential therapy for some disorders has been the use of AOs to alter gene expression through manipulation of pre-mRNA splicing. This concept was initially demonstrated by the suppression of abnormal splicing using AOs to mask cryptic splice sites in the β-globin gene transcript that were responsible for a subset of β-thalassemia cases (Dominski and Kole, 1993, Sierakowska et al., 1996) (Figure 1.5A). To inhibit RNase-H activity, AOs were modified at the ribose moiety at the 2'-OH position on a phosphorothioate backbone (Monia et al., 1993), with this chemistry employed for numerous subsequent studies. As well as using AOs to redirect pre-mRNA processing to restore wildtype splicing patterns, it is possible to target normal splicing to induce exon exclusion or “exon skipping”, such that it bypasses a mutation (Figure 1.5). This could potentially excise an exon containing a premature termination codon, as for the *mdx* model (Figure 1.5B), or skip one or more
exons flanking a frameshift deletion to restore the reading frame, as for the GRMD model (Figure 1.5C).

**Figure 1.5 AO-based modification of pre-mRNA splicing**

(A) Blockage of cryptic splicing as a therapy for β-thalassemia. Mutations within intron 2 of β-globin gene induce usage of cryptic splice sites that incorporate intronic sequence (red box) into the mature mRNA. Disruption of the reading frame introduces a stop codon that results in truncated β-globin protein. Blockage of the cryptic 5' splice site with AO (blue bar) restores normal splicing pattern and functional β-globin protein is produced (Dominski and Kole, 1993). (B) Restoration of dystrophin production in the mdx mouse model...
by exon skipping. A C→T mutation in exon 23 of the mouse dystrophin gene introduces a stop codon that produces a truncated non-functional protein. Blockage of the 5' splice site of exon 23 disrupts its recognition by splicing machinery, resulting in removal of the in-frame exon from the dystrophin transcript. This facilitates translation of near full length, semi-functional dystrophin protein (Mann et al., 2001). (C)

Restoration of dystrophin production in the GRMD model by exon skipping. A point mutation in the acceptor splice site of exon 6 prevents its recognition, inducing its removal from the mature dystrophin mRNA. This disrupts the reading-frame, inducing a premature termination codon in exon 8 that produces a truncated dystrophin. Targeting of exon 6 and 8 with AOs, causes removal of exons 6 and 8/9 such that an internally deleted in-frame transcript is produced to allow translations of a semi-functional protein.

This concept has been applied to DMD where targeting of normal splice sites bypasses mutations that cause premature termination of translation, inducing an internally deleted, yet semi-functional dystrophin protein. For the majority of gene transcripts, removal of an exon or exons would ablate the function of the protein product. For dystrophin though, mildly affected BMD patients with in-frame deletions indicate that some domains are not crucial for near-normal function. The first application of dystrophin exon skipping arose from an observation in a DMD patient that a 52 base-pair (bp) deletion in exon 19 resulted in the splicing of this exon from the mature dystrophin transcript (Matsuo et al., 1991). This suggested that motifs within this region were required for exon inclusion and subsequent targeting of this region with AOs, induced exon 19 removal from normal lymphoblastoid cells in vitro (Pramono et al., 1996).

1.4.1.1 Normal pre-mRNA splicing

To design AOs for efficient exon skipping it is important to first understand the motifs involved in normal pre-mRNA splicing. Whilst the dystrophin gene covers 2.4 Mb of the X chromosome, the coding region only encodes an 11 kb mRNA, meaning that almost 99.4% of the pre-mRNA, consisting of non-coding introns, is removed during splicing (Figure 1.6). This process is catalyzed by a multimeric protein complex that consists of five small nuclear ribonucleoproteins (snRNPs) – U1, U2, U4, U5 and U6, and
Figure 1.6 The process of pre-mRNA splicing

The early (E) complex is formed by binding of U1 snRNP to the 5' donor splice site. The U2 auxiliary factor 35 (U2AF35) binds to the 3' intron acceptor splice site, along with U2AF65, which binds to the pyrimidine tract (Py). The E complex is converted to the A complex upon binding of U2 snRNP to the branch-point (A) through interaction with U2AF 65. A tri-complex of U4-U5-U6 then binds to the 5' splice site (B Complex) before replacing U1, and the spliceosome undergoes rearrangement whereby U4 separates from the U6 snRNP, allowing U6 to bind to U2, resulting in a catalytically active spliceosome (C complex). Splicing can then occur in two transesterification steps; firstly the 2' hydroxyl of the branch-point adenosine attacks the 5'
splice site forming a phosphodiester linkage between the branch-point and 5' terminal nucleotide of the intron, which produces a lariat structure. The newly released 3' hydroxyl of the 5' exon can then break the phosphodiester bond at the 3’ splice site such that a phosphodiester bond is formed between the 5' and 3' exons to form a mature mRNA, whilst concurrently excising the lariat containing the intron and snRNPs (Reed, 1997).

as many as 300 other splicing factors (Jurica and Moore, 2003). These snRNPs have both an RNA component that is complementary to their target sequence, and a protein component that enables its binding to other snRNPs or non-snRNP splicing factors. Assembly of the spliceosome is dependent on conserved sequences within the intron; the 5' and 3' splice sites that define their ends, and the branch-point region that is located 20-40 nucleotides upstream of the 3’ splice site. The process of splicing is detailed in Figure 1.6. Whilst there is a requirement for accurate exon splicing, in higher eukaryotes the intron-exon junctions are defined by weakly conserved intronic cis-elements; the 5’ and 3’ splice sites, and the branch site. Generally, 5’ splice sites are defined by the sequence AG|GURAG (where R is a G or A) and 3’ splice sites by (Y)_{32}NCAG|GN (where Y is a C or U) whilst the branch-point is even less conserved with NNCTVAY (where V is A, C or G) (Cartegni et al., 2002). These sequences are necessary to define intron/exon boundaries but are by no means sufficient to identify genuine splice sites. Sequences that match these sites are very common in introns and define pseudo-exons which although outnumbering genuine exons, are usually not included in the mature mRNA (Sun and Chasin, 2000).

Alternative splicing, whereby several isoforms are produced from the same transcriptional unit, also makes the task of correctly identifying splice sites more complex. Analysis of mRNAs derived from chromosome 22, indicated that approximately 60% of genes had two or more gene transcripts (Lander et al., 2001). Considering also that splicing must be modulated in a developmental and/or cell-type-specific fashion, other mechanisms must be responsible for the recognition of exons in splicing.

1.4.1.2 Exon definition

Exonic enhancer motifs are binding sites for serine/arginine (SR) proteins that have been proposed to be important for exon recognition (Blencowe, 2000). These proteins are a
family of highly conserved and structurally related splicing factors that are characterized by 1-2 RNA recognition motifs (RRM) and a highly enriched Arg/Ser dipeptide domain (the RS domain) (Birney et al., 1993). The RRM's determine substrate specificity through sequence specific binding to the RNA, whilst the RS domains are involved in protein-protein interactions with other splicing factors (Birney et al., 1993). There are two proposed mechanisms for how SR proteins bound to exon splicing enhancers (ESEs) can promote exon definition (Figure 1.7). The first is by directly recruiting splicing machinery through their RS domain, such as U1 and U2AF snRNPs, especially for weakly defined exons (Graveley et al., 2001). An alternate, but not mutually exclusive, proposal is that binding of SR proteins can antagonize nearby silencer elements (Kan and Green, 1999). Further evidence for the importance of ESEs comes from exonic point mutations that result in exon skipping, rather than yielding expected missense amino acid changes (Ars et al., 2000, Teraoka et al., 1999). Additionally, predicted nonsense mutations located within in-frame dystrophin exons that should cause DMD have a BMD phenotype due to exon skipping, presumably indicating that these nonsense mutations are disrupting ESE sites (Disset et al., 2006, Ginjaar et al., 2000).

![Figure 1.7 Models of SR protein interaction with exon splicing enhancers](image)

(A) Binding of the SR protein (light blue) to the ESE can recruit splicing factors such as U2AF35 and U1 snRNP directly, or alternately through indirect action (black arrow) via interactions with the splicing
coactivator Srm160/300m which in turn can interact with U1 and U2 snRNPs. (B) In the absence of SR binding to an ESE, a downstream splicing inhibitor (red) binds to an exonic splicing silencing, preventing splicing of upstream intron. Binding of SR protein counteracts this splicing inhibition. (Adapted from Cartegni 2002)

Early identification of ESEs focused on the study of purine-rich exonic elements, reflecting the preference of some splicing factors for these sites (Cartegni et al., 2002). However, high purine content alone is insufficient to define these regions and precise sequences, which may include interspersed pyrimidines, are likely to be important for binding of numerous and functionally different classes of SR proteins (Cartegni et al., 2002). To predict ESE binding sites, functional SELEX (systematic evolution of ligands by exponential enrichment) assays were performed in vitro (Tian and Kole, 1995) and in vivo (Coulter et al., 1997) to identify sequences that gave good splicing enhancer activity for four different SR proteins (SF2/ASF, SC35, SRp40 and SRp55). This information was then used to produce ESEFinder (rulai.cshl.edu/tools/ESE), a web-based interface to allow computational prediction of the occurrence of exonic enhancers throughout a given sequence (Cartegni et al., 2003).

Exon splicing silencers (ESS) also need to be taken into account when considering exon definition. When short randomly selected human DNA fragments where inserted into the middle exon of a three-exon minigene, approximately one third exhibited splicing inhibitory activity in vivo (Fairbrother and Chasin, 2000). This could suggest that some exons are maintained in a silenced state, and that it is only in the presence of strong flanking splice signals and/or efficient enhancer elements that they are included in the mature mRNA (Cartegni et al., 2002). Although most of the described silencers are intronic, several ESS elements have been reported (Amendt et al., 1995, Zheng et al., 1998). These interact with negative regulators such as the heterogeneous nuclear ribonucleoproteins (hnRNP) family (Dreyfuss et al., 1993), of which the polypyrimidine-tract-binding protein (PTB) is the best characterized (Wagner and Garcia-Blanco, 2001). These inhibitory factors may compete with overlapping enhancer sites such that blockage of a crucial enhancer element may lead to exon silencing. Alternatively, it has been
proposed that binding of PTB to several sites within an exon, and subsequent dimerization of bound PTB, could cause portions of the pre-mRNA to loop out such that it is unavailable for splicing (Blanchette and Chabot, 1999). Exon definition will be dependent on the strength of the flanking splice sites, as well as interplay between positive and negative elements within the exon. The decision therefore, as to whether an exon will be included or not into the mature mRNA, is a complex one. With this in mind, determination of motifs for AO targeting to induce exon skipping will also remain a complex process.

1.4.2 AO chemistries

The use of oligonucleotides for therapeutic applications has become more prevalent in recent times with the advent of RNA interference and therapies to modify pre-mRNA splicing, including exon skipping and translational blockade. Virtually all of the oligonucleotides being developed, or currently on the market or in trial, have some fraction containing modified nucleotides. These modifications are made to either increase stability by improving resistance to serum nucleases, to increase target affinity, to control biodistribution including cellular uptake, or a combination of these. They may also be introduced to the AO to resolve problems associated with affinity for undesired targets and/or potential toxicities (Wilson and Keefe, 2006).

1.4.2.1 2'-O-methyl phosphorothioate

First generation AOs used for gene knockdown studies, including the first antisense drug to be approved by the FDA, Vitravene (Geary et al., 2002), are deoxyoligonucleotides that have all of their non-bridged atoms in the phosphate group replaced with a sulfate group (De Clercq et al., 1969) (Figure 1.8). This phosphorothioate (PS) modification conveys a greatly increased half-life, however these AOs will still induce RNase-H activity when bound to target mRNA (Furdon et al., 1989). In clinical trials with this chemistry, some adverse effects have been observed including activation of the complement cascade (Levin, 1999), and mild to moderate hyperglycemia or hypertension (Chi et al., 2001, Waters et al., 2000). To inhibit RNase-H activity, modification of the 2'-O position of the
ribose with a methyl group (2'-O-methyl) (Figure 1.8) results in an increased affinity for RNA without inducing RNase-H (Sproat et al., 1989). These 20Me PS AOs have been the most commonly employed chemistry to date for DMD exon skipping studies, and are particularly useful for in vitro studies, where they can be effectively delivered into cells in combination with a liposome carrier.

Figure 1.8 Chemical structures of various antisense oligonucleotides

1.4.2.2 Phosphorodiamidate morpholino oligomers

Phosphorodiamidate morpholino oligomers (PMOs) possess a non-ionic backbone whereby the ribose sugar is replaced by a morpholino moiety, and phosphorodiester linkages are replaced with phosphorodiamidates (Summerton and Weller, 1997) (Figure 1.8). They do not elicit RNase-H activity and act through binding to target RNA to sterically block ribosomal assembly or alter pre-mRNA splicing. The uncharged nature of PMOs avoids non-specific interaction with cellular components that have been observed with PS AOs. The modified chemistry also has excellent resistance to nuclease and protease activity, enabling it to have increased stability in plasma and tissues (Hudziak et
al., 1996). One of the disadvantages of the PMO chemistry is that it is taken up poorly in cells in vitro as it cannot be complexed with charge-based delivery systems and as such, physical methods such as scrape loading and osmotic loading have been employed (Ghosh and Iversen, 2000). This could explain why use of PMOs to induce exon 46 skipping in DMD patient cells in vitro was so inefficient (Aartsma-Rus et al., 2004b). Safety evaluation studies in animal models demonstrated that PMOs are well tolerated with no mortality or clinical signs of adverse effects, even with doses several times greater than anticipated therapeutic dose (Amantana and Iversen, 2005). PMOs are currently in phase II/III clinical trials to address a number of disorders including restenosis (Iversen et al., 2003) and Hepatitis C, where almost 250 patients have been administered with no adverse effects reported.

1.4.2.3 Other AO chemistries

Peptide nucleic acids (PNAs) have achiral polyamide backbones consisting of 2-aminoethyl glycine units (Nielsen et al., 1991) (Figure 1.8). Unlike other DNA analogues, they do not contain any sugar moieties or phosphates. Resistance to nuclease and protease digestion confers very high biological stability and they can bind strongly to complementary mRNA with high specificity (Karkare and Bhatnagar, 2006). One of the advantages of PNAs is that the peptide backbone is ideally suited to attachment of receptor ligands or cell penetrating peptides, and these have been demonstrated to enhance PNA uptake in vivo (Fraser et al., 2000, Pooga et al., 1998). Use of PNAs in DMD research has been less successful. A 14-mer PNA targeted to exon 46 in DMD patient cells was unable to induce exon skipping, despite use of a fluorescent label indicating that the PNA was taken into the nucleus (Aartsma-Rus et al., 2004b). An absence of any shift on gel mobility shift assays was suggestive that the PNA was unable to bind to the target sequence. Similarly, application of PNAs to the mdx mouse model in vivo was also unable to induce any skipping of exon 23 from the dystrophin transcript (Fletcher et al., 2006).

Locked nucleic acids (LNAs) are a class of conformationally restricted oligonucleotide analogues, where a ribonucleoside is linked between the 2'-oxygen and the
4'-carbon with a methylene unit (Kumar et al., 1998) (Figure 1.8). The key characteristic for LNAs is their extremely high affinity for DNA and RNA, but they are also non-toxic and nuclease resistant (Wahlestedt et al., 2000). The potential for application to DMD was demonstrated in targeting of exon 46 in DMD patient cells with a 14-mer LNA inducing almost 100% exon skipping in myotubes cultures (Aartsma-Rus et al., 2004b). One of the problems with LNAs though, is that their high affinity for RNA can reduce sequence specificity (Braasch et al., 2002) such that when 1 or 2 mismatches were introduced into the exon 46 LNA, similar or only slightly reduced levels of exon skipping was observed (Aartsma-Rus et al., 2004b). This lack of sequence specificity would have the potential to induce severe side effects in non-targeted genes, especially considering that LNAs would be targeting motifs involved in splicing.

Another recently evaluated chemistry is the chimeric RNA/ethylene bridged analogue that efficiently induced removal of an in-frame exon containing a nonsense mutation, so as to produce a shortened BMD-like protein (Surono et al., 2004). This chemistry has also been previously shown in vitro to be 40-fold more effective than phosphorothioate oligonucleotides in inducing exon 19 removal from the dystrophin gene (Yagi et al., 2004). While this chemistry appears promising, in vivo studies would be necessary to confirm the efficiency and determine any possible adverse effects.

1.4.3 Exon skipping in animal models of DMD

1.4.3.1 Murine studies with 2OMe AOs

As the in-frame exon 23 of the dystrophin gene contains the protein truncating mutation, its removal from the mRNA would bypass the mutation whilst maintaining the reading-frame (Figure 1.5B). This was first demonstrated using a 2OMe 12-mer AO targeting the intron 22 acceptor site to induce skipping of exons 22 to 30, such that dystrophin synthesis was restored in 1-2% of cultured myotubes (Dunckley et al., 1998). Subsequent targeting of the intron 23 donor site induced specific, more consistent and reproducible results at significantly lower concentrations in vitro (Mann et al., 2001, Wilton
et al., 1999) as well as inducing low levels of dystrophin-positive fibres 2 and 4 weeks after treatment in vivo (Mann et al., 2001). Further refinement of design achieved exon skipping at concentrations as low as 10nM in vitro (Mann et al., 2002) and this optimized AO sequence has been used in subsequent studies.

Mice treated with intramuscular injections of 2OMe AO complexed with F127, a non-ionic block copolymer, showed persistent production of dystrophin at near normal levels in large numbers of muscle fibres (Lu et al., 2003). Importantly, this facilitated the localization of the dystrophin associated proteins to the sarcolemmal membrane, as well as partially restoring physiological function as demonstrated by an improvement in maximal isometric tetanic force in treated muscles (Lu et al., 2003). Systemic delivery of 2OMe AOs with F127 via repeated tail vein injection, demonstrated induced dystrophin expression in all skeletal muscles, with highest levels observed in diaphragm, gastrocnemius and intercostal muscles (Lu et al., 2005). Significantly though, no expression was detected in the heart, which is an important consideration for clinical application to humans.

The transgenic hDMD mouse carries an integrated copy of the full-length human dystrophin gene. To determine if exon skipping of this gene was possible in an in vivo setting, 2OMe AOs targeting exons 44, 46 or 49 of the human dystrophin gene were delivered intramuscularly (Bremmer-Bout et al., 2004). One week post-injection, low levels of human dystrophin transcripts lacking the targeted exons could be detected, with no effect on the mouse dystrophin transcript (Bremmer-Bout et al., 2004).

1.4.3.2 Murine studies with phosphorodiamidate morpholino oligomers

PMOs based on optimal 2OMe AO sequence, were delivered to mdx mice using annealed complementary annealed DNA leashes to overcome difficulties associated with transfection (Gebski et al., 2003). Persistent and highly efficient exon 23 skipping was detected in vitro, with high levels of in vivo dystrophin expression detected following a
single intramuscular injection (Gebski et al., 2003). Further in vivo studies undertaken with uncomplexed PMOs demonstrated substantial dystrophin expression following intramuscular injection, with extensive areas of the muscle showing near-normal appearance (Fletcher et al., 2006). In comparison, parallel 2OMe AOs with F127 studies indicated that the PMO chemistry was superior at both the RNA and protein levels (Fletcher et al., 2006). To evaluate PMOs as a potential systemic treatment regimen, repeated intraperitoneal injections were administered to neonatal mice. Dystrophin-positive staining was detected in the diaphragm, ileum and limb muscles, although only low level protein was detectable by western blotting in the tibialis anterior and diaphragm (Fletcher et al., 2006). Systemic delivery of PMO by intravenous injection showed bodywide distribution of dystrophin expression, which was maximal following 7 weekly injections into 2-week old mice (Alter et al., 2006). Western blot analysis showed dystrophin protein levels of up to 50% of normal levels for gastrocnemii and quadriceps, and up to 10-20% for tibialis anterior, intercostals, abdominal and triceps muscles (Alter et al., 2006). Significantly, as in previous studies, no dystrophin expression could be detected in the heart.

1.4.4 Exon skipping in human models of DMD

1.4.4.1 Application to DMD mutations

Exon skipping of the human dystrophin transcript can theoretically be applicable for up to 90% of all DMD patients (Aartsma-Rus et al., 2004a). Exceptions to this are entire gene deletions, large chromosomal arrangements or mutations that are located in regions crucial for dystrophin function. For example; loss of all actin-binding sites, deletions in the cysteine-rich and C-terminal domains, or mutations in the first or last exons of the dystrophin gene, would not be amenable to an exon skipping approach. The first demonstration of restoration of dystrophin protein expression for DMD mutations was in two DMD patient myotubes cultures carrying an exon 45 deletion. Removal of exon 46 using 2OMe AOs targeting purine-rich elements induced ~15% exon skipping, which was sufficient to express properly localized dystrophin in at least 75% of myotubes (van Deutekom et al., 2001). Restoration of dystrophin expression was subsequently
demonstrated in numerous patient-derived cultures including addressing deletions of exon 20 (Takeshima et al., 2001), and within the major deletion hotspot (Aartsma-Rus et al., 2003). For all of these studies, restoration of the reading frame was achieved by targeting a single exon, with dystrophin expressed in 75-80% of patient DMD myotubes.

1.4.4.2 Multiple exon skipping for DMD mutations

Similar to the GRMD mutation, some DMD mutations require removal of two exons to restore the dystrophin reading frame. Targeted removal of a nonsense mutation in exon 43 required simultaneous removal of exon 44 to produce an in-frame transcript in DMD patient cells (Aartsma-Rus et al., 2004a). Efficiency of multiple exon skipping was comparable to single exon skipping, with ~70-75% of transfected myotubes expressing dystrophin. At the RNA level, transcripts missing only one exon were also induced in addition to the in-frame transcripts lacking both exons, though they would be expected to be subject to nonsense-mediated decay (Aartsma-Rus et al., 2004a). Targeted AO removal of multiple exons around genomic deletions has the potential to induce in-frame deletions that could mimic known BMD genotypes. This was demonstrated by application of 2OMe AOs targeting exon 44 and exon 51, to an out-of-frame exon 46-50 genomic deletion, which induced an in-frame transcript that spliced exon 44 to 52, such that dystrophin protein was produced (Aartsma-Rus et al., 2004a). A strategy to induce larger in-frame deletions than the minimum requirements to overcome a DMD mutation may be preferable. By targeting a large stretch of exons, multiple DMD mutations could be addressed using the same AO treatment. In the previous example, removal of exon 45-51 would be beneficial for over 13% of all DMD patients reported in the Leiden database (Aartsma-Rus et al., 2006a). To explore the feasibility of inducing large multi-exon skips, 2OMe AOs were used to target the boundary exons for exon 17-48, 17-51, 42-55 and 48-59 deleted regions (Aartsma-Rus et al., 2006a). Multi-exon skipping was not observed for exons 17-48, 17-51 or 48-59 in either control or patient myotubes cultures. For exon 42-55, multi-exon skipping was inconsistent, working in only 4% of experiments, and at a low level such that dystrophin protein could not be detected in cultures derived from a DMD patient with an exon 46-50 deletion (Aartsma-Rus et al., 2006a). Whilst large multi-exon skipping does not appear feasible from these studies, targeting of each individual exon with a cocktail of
AOs was able to induce the removal of exons 19-25 in the mdx mouse model (Fall et al., 2006). AO design was optimized using 2OMe AOs in vitro, prior to application in vivo using PMOs. Shortened transcript corresponding to deletion of exons 19-25 could be detected in vivo, with dystrophin protein detectable for up to 8 weeks following a single injection (Fall et al., 2006). This study suggested that whilst targeting the outermost exons may be insufficient to induce removal of large multi-exon regions, a cocktail of AOs directed against each exon could allow this approach to be feasible.

Exon skipping has also been applied to address duplication mutations. Targeting of an exon 45 duplication with 2OMe AOs revealed significant levels of single exon 45 skipping, with low levels of double exon 45 skipping (Aartsma-Rus et al., 2007). Consequently, low levels of dystrophin protein (~8% of normal) could be detected 5 days after transfection in patient myotubes (Aartsma-Rus et al., 2007). When targeting an exon 44 duplication however, no dystrophin could be detected due to high levels of double exon 44 skipping, even at low AO concentrations, that induced an out-of-frame transcript (Aartsma-Rus et al., 2007). To overcome this, simultaneous targeting of exon 43 induced transcripts missing exon 43 and both exon 44’s, such that an in-frame transcript was produced, and dystrophin observed at 3% of normal levels (Aartsma-Rus et al., 2007). Attempts to induce removal of a larger exon 52-62 duplication by targeting of exons 52 and 62 were unsuccessful, with difficulties associated with assaying exon skipping of such a large region from the dystrophin transcript. However, absence of any dystrophin suggests that as with previous multiple exon skipping studies, targeting of the outermost exons of a large region may not be sufficient for exon removal.

1.4.4.3 Studies of AO design

Initial exon skipping studies in the mdx mouse suggested that consensus splice sites should be targeted for exon removal (Dunckley et al., 1998, Mann et al., 2002). Subsequent studies in targeting exons of the human dystrophin gene highlighted the importance of ESEs in determining exon inclusion to the mature mRNA. In a retrospective analysis of 114 AOs that were developed for skipping of 36 exons, of which two-thirds
were effective, a number of conclusions about AO design were made (Aartsma-Rus et al., 2005). The first was that the “openness” of secondary structure, as determined by in silico prediction, was no different between effective versus ineffective AOs (Aartsma-Rus et al., 2005). The second was that effective AOs targeted significantly more ESEs, as predicted with the use of ESEFinder 2.0, than ineffective AOs did (Aartsma-Rus et al., 2005). Effective AOs were also located significantly closer to the acceptor splice sites, which correlates with studies that report ESEs located within 70 nucleotides of the acceptor splice site are more active than ESEs further away (Fairbrother et al., 2004, Wu and Maniatis, 1993). In a comprehensive study of human dystrophin exon skipping, 2OMe AOs were developed for targeted removal of each individual exon of the dystrophin gene, except for the first and last (Wilton et al., 2007). Again there was a general tendency for the most effective AOs to target either acceptor sites or ESEs in the first half of the exon. No single motif, whether consensus splice sites, or putative ESEs, was identified as a reliable target for consistent exon removal, although the majority of effective AOs targeted internal exonic regions (Wilton et al., 2007). For a subset of exons, two or more AOs were necessary to induce exon skipping. This did not appear to be due to cumulative AO activity, as no exon skipping could be detected when targeting the exon with either individual AO (Wilton et al., 2007), but presumably due to a need to target multiple ESEs to interfere with exon recognition. In further analysis of AO cocktails, targeting of exon 65 with individual AOs could not induce its removal, whereas for some cocktails, highly efficient skipping (20% at 5nM) could be obtained, but for other AO combinations, exon skipping could not be induced (Adams et al., 2007). The most highly effective AO cocktails did not necessarily block acceptor and donor splice sites, neither did they correlate with the number of putative ESEs targeted, although there was a trend for targeting of SC35, but this was not absolute (Adams et al., 2007).

With regards to the importance of AO length, targeting of exon 23 in the mdx mouse indicated that increasing AO length from a 20-mer to a 25-mer induced more efficient and persistent exon skipping in vitro (Harding et al., 2007). Similarly, in human studies of exon 16 skipping, increasing a 25mer AO by six nucleotides in either the 5’ or 3’ direction, substantially improved exon skipping efficiency (Harding et al., 2007). Interestingly, a
20mer AO that targeted motifs common to both of the efficient 31mers, was unable to induce any exon skipping (Harding et al., 2007). This is further evidence that targeting of more than one splicing motifs may be necessary to inhibit exon recognition.

1.4.5 AO delivery

The efficient administration of AOs to the relevant organs or tissues expressing dystrophin is recognized as one of the greatest challenges to the implementation of AO induced exon skipping. As well as for skeletal and cardiac muscle, AOs may have to address other dystrophin isoforms that are expressed in non-muscle tissues including the brain. Whilst delivery of AOs can be achieved readily *in vitro* with the use of cationic polymers, such as Lipofectamine or poly-ethylenimine (PEI), efficient AO delivery *in vivo* is significantly more difficult. A number of barriers must be overcome for effective nucleic acid delivery to the cell, including degradation in the extracellular space, internalization, escape from endosomes into the cytoplasm, dissociation from the carrier and transfer into the nucleus (Roth and Sundaram, 2004). For DMD, local injection into the muscle is the most straightforward approach; however it is unlikely to be feasible in a clinical setting where there is a limited spread of dystrophin expression and where almost 30% of body mass is muscle, with many muscles not easily accessible without surgery. Additionally, as AOs have a limited half-life, any delivery technique must be able to facilitate readministration of AOs. Taking these factors into account, systemic delivery is likely to be the only option for application to the entire muscular system. Systemic delivery of PS AOs by intravenous injection results in the majority of AOs being taken up by the liver and rapidly cleared from the body, with a plasma elimination half-life as low as 38-75 minutes reported (Sereni et al., 1999). In comparison, the half-life of PMOs has been estimated to be about 10 hours from clinical trials of a *c-myc* antisense PMO (Arora et al., 2004). Given that AOs are rapidly degraded in plasma, or excreted through the urinary system, it is important to maximize the uptake of AOs into the target cells in the short time that they are available. To enhance systemic AO delivery and uptake, a number of avenues of research are being explored.
1.4.5.1 Cationic systems

Uptake of anionic nucleic acid, such as 2OMe AOs, is relatively poor due to electrostatic repulsion against the negatively charged cell surface. Naked AOs also have a tendency to localize into endosomes/lysosomes where they are not able to exert their antisense effects. To overcome both of these issues, cationic polymers or liposome systems have been used to generate positively charged complexes to facilitate entry into the cell and subsequent release into the cytoplasm. Although used successfully to efficiently transfect AOs in vitro, including in numerous DMD studies, in vivo application has proved more difficult. Positively charged cationic complexes can bind to anionic serum proteins, which in turn can lead to toxicity and instability of the complex (Thierry et al., 2003). One such carrier is PEI, a cationic polymer that can form PEI-nucleic acid complexes that appear to be taken up into the cell by non-specific endocytosis and subsequently released from endosomes through the protonation of amino groups within the PEI (Akinc et al., 2005). Incorporation of polyethylene glycol (PEG) polymers, forms a core-shell structure (Petersen et al., 2002) which improves functionality by providing improved solubility, lower surface charge, reduced aggregation and importantly, lowers toxicity by shielding the surface charge (Sung et al., 2003) of the PEI complex. However, these modifications have also been shown to lower transfection efficiency (Kichler et al., 2002). Although PEI-PEG complexes have been used primarily for plasmid DNA delivery, there is strong evidence that they can be adapted for delivery of oligonucleotides (Fischer et al., 2004, Jeong et al., 2003, Kunath et al., 2002). Indeed, application of PEG-PEI to the mdx mouse by intramuscular injection resulted in widespread dystrophin expression with no apparent cytotoxicity (Williams et al., 2006). Further investigation will be necessary to determine if this success can be replicated for systemic delivery.

1.4.5.2 Nanoparticle systems

Nanoparticles are submicronic (<1 μm) colloidal systems made of polymers that can be used to form nanocapsules in which the drug or nucleic acid is confined to an oily or aqueous core, surrounded by a thin polymeric membrane (Toub et al., 2006). Early nanoparticle systems used for drug delivery where taken up by the mononuclear phagocyte...
system, where they were taken into the lysosomes and degraded, releasing the compounds into the cell (Lenaerts et al., 1984). For application to AO delivery, cationic peptides have been added to the nanoparticles to destabilize the lysosome membrane to allow AO release into the cell (Chavany et al., 1994). Likewise, linkage of PEG polymers to the nanoparticle can decrease uptake by phagocytes, such that they have a longer circulation time (Gref et al., 1994). Although nanoparticle-mediated delivery has not been applied to AO exon skipping for DMD, it has been used to demonstrate improvement in AO stability in vivo as well as increased tissue uptake, particularly to the liver (Nakada et al., 1996). As nanoparticle membranes can be highly modified, there is also the potential for target specific ligands to be used for systemic delivery to the muscle, although this has yet to be demonstrated.

1.4.5.3 Cell penetrating peptides

Cell penetrating peptides (CPPs) are short (<30 amino acid) cationic and/or amphipathic peptides that have been used to facilitate delivery of a wide range of cargoes, including proteins and peptides, antisense nucleic acids, siRNAs and plasmid DNA (reviewed in (Mae and Langel, 2006)). These peptides were originally derived from naturally occurring proteins with membrane translocation activity, such as penetratin from Drosophila (Derossi et al., 1994), and the Tat peptide from HIV-1 TAT protein (Vives et al., 1997). Since then, multiple CPP sequences have been identified and synthesized including transportan (Pooga et al., 2001), HSV-1 protein VP22 (Elliott and O'Hare, 1997) and polyarginine (Futaki et al., 2001). Their potential for in vivo application was first demonstrated by intraperitoneal administration of a TAT-βgal protein, resulting in delivery to all tissues of the mouse, including the brain (Schwarze et al., 1999). Although the exact mechanism of cellular uptake by CPPs is unknown, they have been shown to rapidly induce endocytosis upon binding to the cell membrane, thought to be due to intracellular signaling pathways that induce membrane ruffling (Wadia et al., 2004)

CPPs have been linked to both PNA (Shiraishi and Nielsen, 2006, Shiraishi et al., 2005) and PMO (Moulton et al., 2004, Nelson et al., 2005) AOs to modify pre-mRNA
splicing by blocking cryptic splice sites in a human β-globin luciferase system that restored normal splicing to allow translation of luciferase. Success with an arginine-rich peptide (R9F2) used in the PMO studies, led to its utilization for exon skipping in the mdx mouse model. PMOs directed against exon 23, linked to R9F2, were shown to induce highly efficient exon skipping in vitro, and subsequent widespread dystrophin expression and near-normal muscle architecture, except in the heart, following intraperitoneal injection (Fletcher et al., 2007). This CPP was subsequently used in studies in this thesis for application to the GRMD animal model and human cellular models of DMD.

1.4.5.4 Viral vectors

Although AOs are good candidates for clinical trials, due to their low toxicity and the ease with which treatment can be halted if any adverse effects are encountered, readministration will be necessary as these therapeutic compounds have a limited biological half-life and will transiently target the gene transcript rather than the gene. To address this limitation, recombinant vectors were constructed to stably express large amounts of modified small nuclear RNAs (snRNAs) containing antisense sequences in vivo. The action of the antisense sequence is enhanced when linked to snRNAs, which facilitates proper subcellular localization and inclusion into the mRNA splicing process (De Angelis et al., 2002). The first report of this strategy for the dystrophin transcript was the targeted removal of exon 51, to restore the reading frame in a patient cell line missing exons 48-50 (De Angelis et al., 2002). This study used a number of constructs incorporating both U1 and U7 snRNA elements where the most efficient construct had a U7 snRNA natural histone spacer element antisense sequence replaced with sequence specific to both the 5’ and 3’ intron/exon junctions of exon 51. Transfection of cultured myoblasts with the construct was able to induce exon 51 removal from the dystrophin transcript and subsequent production of near full length dystrophin.

Administration of rAAV vectors expressing antisense sequences targeted to dystrophin exon 23, linked to a modified U7 snRNA, induced sustained high levels of dystrophin production in the mdx mouse (Goyenvalle et al., 2004). Following a single
intramuscular injection, almost all fibres in the muscle expressed dystrophin after 4 weeks. This dystrophin expression also coincided with functional improvement as measured by resistance to tetanic contraction damage, and the ability to resist exercise induced injury. Intra-arterial perfusion of the lower limb also resulted in the rescue of >80% of the fibres in most muscles of the perfused leg. The effectiveness of this approach was confirmed using U1 snRNP rAAV vectors, with similar results to those obtained previously (Denti et al., 2006). Continuous stable production of AOs would forgo the need for repeated administration; however the use of viral vectors may evoke some of the shortcomings associated with conventional viral gene therapy such as high cost, immunological reactivity and random integration into the genome. This strategy has also been applied for an ex vivo gene therapy approach, where human muscle precursor cells from a DMD patient with an exon 49-50 deletion were transfected with an exon 51 U7snRNA lentiviral vector (Quenneville et al., 2007). Restoration of dystrophin was observed in 30% of dystrophin transcripts in vitro and human dystrophin could be detected 10 weeks following transplantation into SCID mice, albeit at low levels in only a few myofibres.

### 1.4.6 Clinical application

Since the initial application of exon skipping in 1996 to bypass DMD mutations, this field of research has moved relatively rapidly such that proof-of-principle clinical trials have been recently completed. The first clinical application of AOs to induce exon skipping was the intravenous infusion of a phosphorothioate AO into a 10-year-old patient with a deletion of exon 20 (Takeshima et al., 2006). The patient received four doses of 0.5mg/kg body weight of AO directed against exon 19 at one week intervals. No apparent adverse reactions were observed during the treatment period. However, serum creatine kinase levels, which are a marker of muscle damage, were not reduced during the treatment. RT-PCR analysis demonstrated low levels of exon 19 skipping in both lymphocytes and skeletal muscle, with approximately 6% of muscle dystrophin transcripts missing exons 19-20. Dystrophin could be detected in muscle biopsies localized to the sarcolemma, however staining was patchy and at a very low level. Based on these results it is not surprising that no apparent muscle strength improvement could be observed (Takeshima et al., 2006). One of the problems with this clinical trial was the choice to use
phosphorothioate AOs, which could potentially induce RNase-H degradation of the dystrophin transcript and hence, reduce the efficacy of the AO to induce exon skipping.

Preparations are currently underway in the United Kingdom for a phase I/IIa clinical trial to assess the safety and effect of locally administered AOs directed against exon 51. This study will parallel a trial that has been recently completed by the Leiden DMD group in collaboration with Prosensa, whereby four boys received a single injection into a small area of the tibialis anterior muscle of 0.8mg of 2OMe AO directed against exon 51. Although this trial has been completed, the results have yet to be published, however conference reports indicate that no adverse effects were observed and dystrophin-positive fibres could be detected around the site of injection (personal communication). For the UK trial, a similar protocol will be employed, but instead of 2OMe AOs, PMOs targeting exon 51 will be used. The optimal AO sequence for exon 51 skipping was determined by comparing eight different sequences in a blinded fashion, using normal and DMD cell cultures, as well as muscle explants (Arechavala-Gomeza et al., 2007). Should these initial trials prove successful they will be extended to assess the efficacy of systemic administration of PMOs to DMD patients.

1.5 OUTLINE OF THESIS

At the commencement of this thesis in 2003, relatively few studies had been published in the field of exon skipping as a therapy for DMD. Most of these studies focused on the use of the mdx mouse model, which as stated previously, has only a mild dystrophic phenotype. The GRMD model, in comparison, has a phenotype that more closely resembles the pathology and clinical progression of DMD. As such, this model may be preferable for testing of potential therapies for DMD prior to clinical application in patients. As exon skipping had been successful demonstrated in the mdx mouse and in DMD patient cultures, we hypothesized that an AO-mediated exon skipping approach could be applied to the GRMD animal model to provide a platform for testing of AO chemistries
and delivery methods using this approach. To bypass the GRMD mutation it would be necessary to induce the simultaneous removal of exons 6 and 8 from the GRMD transcript such that an in-frame, internally-deleted dystrophin protein was produced. Optimisation of this AO exon skipping approach was performed in vitro in GRMD derived myotubes cultures and is presented in Chapter 2. In Chapter 3 this approach was extended to in vivo administration, with three different AO chemistries tested for their ability to induce dystrophin protein following localized injection to a single muscle.

Whilst animal models are useful for demonstration of proof-of-principle, testing of efficacy, and addressing issues of safety for a new therapeutic approach, the importance of in vitro models of the human condition cannot be underestimated. In previous studies in DMD patient-derived cells, the exon skipping strategy utilized only the minimal exon removal necessary to bypass the DMD mutation. However, for many phenotypically mild BMD patients, the presence of larger multi-exon deletions suggests that induction of these genotypes may be preferable. Induction of a large multi-exon skipped region may also be preferable for clinical applications whereby a “cocktail” of AOs could address multiple DMD mutations within this region with a more consistent functional outcome. Studies presented in Chapter 4 were therefore performed to test the feasibility of an exon skipping approach to induce the removal of a large multi-exon region of the dystrophin transcript, namely from exons 3-9.

Human exon skipping studies had previously only been performed in myotube cultures derived from DMD patient muscle biopsies. Whilst useful for demonstrating the feasibility of an exon skipping approach, these in vitro systems are significantly different from the complex three-dimensional structure of skeletal muscle. Development of the hDMD mouse provided a model to test human dystrophin exon skipping in an in vivo context; however this was on a mouse splicing background which may not be ideal. It was therefore hypothesized that an ex-vivo system could be used to demonstrate exon skipping in a model that was a truer representation of the human in vivo situation. This work is
presented in Chapter 5, where muscle explants derived from normal and DMD tissue biopsies were used to test the efficacy of exon skipping using different AO chemistries.

Overall this thesis provides a significant and novel contribution to the field of DMD research, specifically in the use of AOs to modify pre-mRNA splicing to overcome DMD mutations. Studies in this thesis demonstrate the first application of an exon-skipping approach to the GRMD animal, which should facilitate the use of this model for pre-clinical testing of any AO chemistries or delivery systems that may arise. By investigating the limitations of exon skipping through targeted removal of large regions of the dystrophin transcript, the options for clinical application of this approach will be better understood. Finally, demonstration of exon skipping in another DMD model provided further evidence for the potential of this approach as a therapy for DMD.


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Chapter 2

Antisense oligonucleotide induced exon skipping restores dystrophin expression \textit{in vitro} in a canine model of DMD

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2.1 Introduction

Duchenne muscular dystrophy (DMD) is a serious X-linked recessive myodegenerative disease typically caused by nonsense or frame-shifting mutations in the dystrophin gene that result in an absence of functional protein (Hoffman et al., 1987). The precise molecular roles of dystrophin are still being elucidated, but primarily it appears to stabilise the myofibre membrane. An absence of dystrophin compromises the link between the myotube cytoskeleton and the sarcolemmal complex, thereby increasing muscle fragility and leading to a continual degeneration of muscle fibres. Until recently, death occurred due to cardiac or respiratory failure typically before the third decade (Emery, 2002), but new treatments and patient management, including glucocorticoid regimens and assisted ventilation, have made substantial improvements (Mellies et al., 2003, Alman, 2005).

Becker muscular dystrophy (BMD) arises from mutations in the dystrophin gene that typically cause internal in-frame deletions, resulting in a shortened but still partially functional dystrophin protein. In some cases, the internally truncated protein appears to fulfill the role of normal dystrophin so that there are no obvious clinical symptoms and diagnosis may only occur later in life (Heald et al., 1994).

One proposed strategy for the treatment of DMD is the application of antisense oligonucleotides (AOs) to remove specific exons from the dystrophin mRNA transcript
during pre-mRNA processing to either bypass nonsense mutations or to restore the mRNA reading frame around a genomic deletion or duplication. If successfully applied, a DMD gene transcript could be modified so that it may eventually express a BMD-like protein, where manipulation of pre-mRNA splicing could potentially convert a severe DMD phenotype to a milder form of the disease. This approach has been applied to the mdx mouse model, both in vitro and in vivo (Mann et al., 2001, Lu et al., 2005) and to normal and DMD patient cell lines (van Deutekom et al., 2001, Aartsma-Rus et al., 2003). In the mdx mouse, AOs were targeted to remove the in-frame exon 23 and hence the mdx nonsense mutation, such that a shortened but semi-functional protein was produced (Mann et al., 2001). In studies in human DMD cell lines, AOs were used to target single and multiple exons to restore the dystrophin mRNA reading-frame such that a BMD-like protein was induced (Aartsma-Rus et al., 2004a). Here, we report the evaluation of this approach to restore dystrophin expression in the golden retriever muscular dystrophy (GRMD) animal model.

Unlike the mdx mouse, which superficially appears clinically normal throughout most of its life, the GRMD dog may be regarded as a more relevant model to study DMD. Affected dogs suffer a fatal, muscle wasting clinical course with fibrosis, contractures and weakness similar to DMD (Howell et al., 1997). As such, the similarities in disease progression, and size of the dogs, should be more appropriate in assessing delivery and efficacy of AOs to address dystrophin mutations before moving to human clinical trials.

The genetic basis for GRMD is a point mutation in the consensus splice acceptor site in intron 6 of the canine dystrophin gene (Sharp et al., 1992). This results in the removal of exon 7 from the mRNA transcript causing a frame-shift, such that a prematurely truncated non-functional protein is generated. The development of a treatment for GRMD dogs has relevance to the human disease since exon 7 is within a minor deletion hotspot of the human dystrophin gene. Furthermore, splice site mutations in the human dystrophin occur at a frequency of some 10-15% and the consequences of a frame-shift in the mRNA is analogous to the more common genomic deletions encountered in DMD.

We investigated the efficacy of using antisense-induced exon skipping as a potential treatment for GRMD, where AOs were optimized to target splicing motifs to induce the
removal of exons 6 and 8 from the dystrophin mRNA to restore the reading frame. A comparative analysis of the efficacy of 2'-O-methyl phosphorothioate (2OME), phosphorodiamidate morpholino oligomers (PMOs) and peptide linked PMOs (PMO-Pep) chemistries to induce dystrophin protein was performed in vitro. AOs have been more commonly used to down-regulate gene expression through RNase-H mediated degradation (Crooke, 1999). However, the chemistries used here do not support RNase-H activity, and in this application rely upon preventing correct assembly of the splicing machinery, through either a displacement mechanism or alteration of essential secondary structure. Whilst 2OME and PMO chemistries have been used previously in both mouse and human exon skipping studies (Gebski et al., 2003, Aartsma-Rus et al., 2004b), here we report the first use of peptide linked PMOs to induce modified splicing in the GRMD dystrophin gene transcript. One of the major limitations in evaluating PMOs in cultured cells has been their poor uptake and hence lack of apparent activity (Aartsma-Rus et al., 2004b). To overcome this problem, PMOs have been conjugated with arginine-rich transport peptides to enhance their cellular uptake (Moulton et al., 2004). Peptide-conjugated PMOs (PMO-Peps) were compared to non-conjugated PMOs and 2OME AO of identical sequence for their ability to modify the GRMD transcript. AO chemistries used in this study are shown in Figure 2.1. This study provides proof of principle that this AO approach can be used as a therapy for DMD in a more clinically relevant animal model and provides further insight into the most suitable choice of AO chemistry for future studies.

2.2 Materials and Methods

2.2.1 AOs and Primers

The nomenclature and sequences of 2OME AO, PMOs and PMO-Pep are shown in Figure 2. 2OME AOs were synthesized in house on an Expedite 8909 Nucleic Acid Synthesizer using the 1μmol thioate synthesis protocol with 2'-O-methyl cyanoethyl phosphoramidites and support columns supplied by Glen Research, Sterling, USA. Upon completion of synthesis, the AO was deprotected and cleaved from the column support with NH₄OH. AOs were desalted using NAP-10 columns under sterile conditions. All 2OME AOs have a phosphorothioate backbone with a 2'-O-methyl ribose modification. AOs were designed complementary to target sequences for canine dystrophin exon 6 and exon 8 and
their respective introns (Figure 2.2). Nomenclature system for the AOs was based on that
used in the mdx mouse (Mann et al., 2002) where C indicates canine, # refers to exon and
(#:/#) refers to the annealing co-ordinates relevant to the intron/exon boundary. PMOs and
PMO-Pep conjugates of identical sequence to the optimised 2OMe AOs were synthesized
by AVI BioPharma, Corvallis, USA. Primers for RT-PCR analysis were synthesized by
Geneworks, Adelaide, Australia.

2.2.2 Cell culture and AO transfection
Muscle biopsies were obtained from affected pups within the GRMD colony
maintained at Murdoch University, West Australia. Primary myoblasts were isolated by
enzymatic dissociation using a DMEM solution containing 2.4U/ml dispase (Roche), 1%
w:v type II collagenase (Roche) at 2ml/g of tissue incubated at 37°C for 45min. Myoblasts
were proliferated at 37°C, 5% CO₂ in Hams F-10 media (Invitrogen) supplemented with
20% fetal bovine serum (Invitrogen), 0.5% chick embryo extract (US Biological), 10U/ml
penicillin (Invitrogen), 10μg/ml streptomycin (Invitrogen) and 250ng/ml amphotericin B
(Sigma). Myoblasts were then plated at 2x10⁴ cells/well into 24-well plates for RNA
analysis or at 1x10⁶ cells into a 25cm² flasks for protein analysis. Plates were coated with
50μg/ml poly-D-lysine (Sigma) and 100μg/ml Matrigel (Becton Dickinson) and flasks with
Matrigel alone. Myoblasts were differentiated in DMEM supplemented with 5% horse
serum (Invitrogen) for 3 days for RNA studies and 14 days for protein studies prior to
transfection. 2OMe AOs were transfected in Opti-MEM using Lipofectamine 2000 (L2K)
at a 1:1 (wt:wt) AO:lipoplex ratio according to manufacturer’s instructions (Invitrogen)
whilst PMO and PMO-Pep conjugates had no delivery agent and were transfected in
DMEM with 5% horse serum.

2.2.3 RNA extraction and RT-PCR analysis
RNA was extracted from pooled duplicate wells of AO transfected cells using
Trizol (Invitrogen) at time points specified. RT-PCR was performed with 100ng of total
RNA for 35 cycles of amplification using 1U of Superscript III (Invitrogen) in a 12.5μl
reaction volume. Primers amplifying from exons 1 to 10 (CanDys1Fa, 5’ -
catcagagaaaaacgaatagg -3’ and CanDys10Ra, 5’ - aatctctcttmtgtgctcag - 3’) were used at
94 °C for 30 sec, 55°C annealing for 1min, 72°C extension for 2min. A 1μl sample from
this reaction was then used as the template for 30 cycles of secondary PCR amplification using 0.5U of AmpliTaq Gold (Applied Biosystems). Nested PCR was performed using primers for exons 1 to 10 (CanDys1Fb, 5' – gttggaagaagtagaggactg – 3' and CanDys10Rb 5' – ctcagctgaagaagccacga – 3') under cycling conditions described above. Products were then electrophoresed on a 2% TAE agarose gel, with products of interest purified using UltraClean spin columns (MoBio) and then sequenced on an Applied Biosystems 377 automated sequencer using BigDye V3.1 terminator chemistry (Applied Biosystems).

2.2.4 Protein extraction and Western blotting

Protein was extracted from AO-treated T-25 flasks 7 days following transfection. Cells were trypsinised, pelleted by centrifugation and resuspended in 300μL of protein extraction buffer [125mM Tris-HCl, pH 8.8, 4% (w:v) SDS, 40% (v:v) glycerol, 0.5mM PMSF and 0.5μl protease inhibitors cocktail (Sigma)], sonicated and heated to 95°C for 5min. Prior to protein extraction, 10% of cell suspension was used for RNA extraction and subsequent exon skipping analysis. 50μL of extracted protein volume was loaded onto a denaturing 3-10% gel with a 3% stacking gel and electrophoresed at 30mA for 5h at RT. As a positive control, 10 and 30μL of protein extracted from 14 day myoblast cultures from unaffected golden retriever littermates was used. Fractionated proteins were then electroblotted overnight at 290mA at 18°C onto nitrocellulose membrane (Amersham). DYS 1 (Novacastra) antibody (1:100) was used to probe for the presence of canine dystrophin and NCL-SPEC2 (Novacastra) antibody (1:100) for β-spectrin. Chemiluminescent detection was performed using Western Breeze (Invitrogen) according to the manufacturer’s instructions. Membranes were then exposed to Hyperfilm (Amersham) for visualisation.

2.3 Results

2.3.1 AO Design for skipping exons 6 and 8

The removal of either exon 6 or exon 8 alone from the GRMD dystrophin mRNA transcript will not restore the reading frame disrupted by the loss of exon 7. The simplest solution to restoring the reading frame is the simultaneous removal of exons 6 and 8 such that an internally deleted protein missing 159 amino acids would be produced (Fig 2.3A).
An alternate approach would be the removal of exons 8, 9, 10 and 11. The simpler option of targeting two exons was chosen and 2OMe AOs were used to optimize the target site for AO induced skipping of exons 6 and 8.

Previous studies in the *mdx* mouse indicated that the 5' donor splice site of exon 23 was the most amenable target to induce 23 exon skipping. 2OMe AOs were designed to the donor splice site for both exon 6 [C6D(+12-13) and C6D(+07-19)] and exon 8 [C8D(+02-15) and C8D(+08-17)] (Figure 2.2). Transfection of these AO lipoplexes at 300nM failed to induce exon skipping for either exon as determined by nested PCR analysis (Fig 2.3B). Constitutive skipping of the in-frame exon 9 (132bp) was observed in all samples including untreated and L2K only treated cells and has been reported elsewhere (Reiss and Rininsland, 1994). Another series of 2OMe AOs were subsequently designed to target the 3' acceptor splice sites of introns 5 [C6A(-14+06) and C6A(-10+10)] and 7 [C8A(-10+10) and C8A(-04+18)] respectively (Figure 2.2). Although no exon 6 skipping was observed, C8A(-04+18) was able to induce efficient skipping of exon 8 (185bp) from the mRNA transcript at 300nM (Fig 2.3B) while C8A(-10+10) was found to be ineffective under these conditions. The removal of exon 8 was always accompanied by the removal of the in-frame exon 9, as confirmed by DNA sequencing analysis (data not shown). This was further confirmed by amplification across exons 1-9 which indicated that transcripts skipping exon 8 alone could not be detected as only full length product was observed (Fig 2.3C).

As targeting of exon 6 consensus splice sites had failed to induce exon skipping, additional 2OMe AOs were designed to target putative exon splicing enhancer (ESE) elements identified using ESE Finder version 2.0 (http://rulai.cshl.edu/tools/ESE/) (Cartegni et al., 2003). Targeting a putative exon 6 ESE site with the AO [C6A(+69+91)] induced efficient removal of exon 6 (173bp) from the dystrophin transcript (Fig 2.3B). Most transcripts missing exon 6 also had exon 9 absent. Since transcripts missing exon 9 were detected in untreated or sham treated cultures, it is assumed that the removal of exon 9 was a constitutive event and was not influenced by the AO. However, unlike the induced skipping of exons 8 and 9, the removal of exon 6 alone could be observed in amplification from exons 1-9 (Fig 2.3C). To determine the efficacy of these AOs, C8A(-04+18) and C6A(+69+91) were transfected at a series of concentrations from 20nM to 600nM. For
both C6A(+69+91) (Fig 2.3D) and C8A(-04+18) (Fig 2.3E), removal of exon 6 and exon 8 was consistent and pronounced to 50nM with lower levels of exon skipping still observed at 20nM. This induced skipping also resulted in reduced levels of the full length transcript at higher AO concentrations.

2.3.2 Exon 6 and 8 removal to restore mRNA reading frame

Having designed AOs to induce the removal of exons 6 and 8, PMO and PMO-Peps of identical sequence were synthesized. Canine myoblasts were co-transfected over a range of concentrations using equal amounts of C6A(+69+91) and C8A(-04+18) for each AO chemistry to allow a comparison of AO efficacy. Transfection with 20Me AO was limited by AO:lipoplex associated toxicity at higher concentrations. A delivery agent for 20Me AO transfection was essential as uncomplexed 20Me AOs, even at 10μM concentration, induced no detectable exon skipping (data not shown). The uncharged PMO and PMO-Peps could not be complexed with the cationic liposome but were transfected alone and showed no obvious deleterious effects on the cultured cells at any concentration tested. Three days after transfection, simultaneous removal of both exons from the mRNA transcript was observed for all AOs tested, with no induced skipping in untreated samples (Fig 2.4). As anticipated, exon 9 was also removed so that the AO-induced dystrophin transcript was missing exons 6-9 (466bp). The precise splicing of exon 5 to exon 10 was confirmed by sequencing analysis (Fig 2.4D) and represents an in-frame transcript which should be translated into a semi-functional protein. RNA analysis of downstream dystrophin mRNA did not show any aberrant splicing indicating that induced exon skipping was specific to the region targeted (data not shown). Removal of either exon 6 or exon 8/9 from the mRNA transcript alone was also observed for all AO chemistries (Fig 2.4). Transfection with 20Me AO lipoplexes induced removal of exons 6-9 at 20nM, albeit weakly (Fig 2.4A). In contrast, uncomplexed PMO only induced high levels of 6-9 skipping at 20μM with weak exon skipping at 10μM (Fig 2.4B). However, the use of the conjugated peptide improved exon skipping, such that high levels of 6-9 removal was observed at concentrations of 300nM with removal of either 6 or 8/9 alone also observed at 50nM (Fig 2.4C). Significantly, essentially no product representing the intact dystrophin transcript was detected in those cells transfected at or above 300nM (Fig 2.4C).
2.3.3 Persistence of in-frame transcript

Whilst each of the 2OMe, PMO and PMO-Pep combination of AOs had induced the in-frame transcript, we sought to determine the persistence of induced exon skipping for each chemistry. PMO and PMO-Pep were transfected at 10μM, 5μM and 1μM and the 2OMe AO lipoplexes at 300nM, 150nM and 75nM (Fig 2.5). RNA was extracted 1, 4, 7 and 10 days following a single transfection and RT-PCR analysis performed. 2OMe AO lipoplexes initially induced high levels of exon skipping but this rapidly declined by day 7 and was only detected at low levels on day 10 at the highest transfection concentration (Fig 2.5D). It should be noted this coincided with a reduction in cell number from day 7 onwards, presumably arising from AO lipoplex associated toxicity. Transfection with PMOs induced negligible levels of transcript missing exon 6-9 from days 1 to 10, although low levels of transcripts with exon 6/9 (636bp) or 8/9 (624bp) exclusion were present (Fig 2.5). Transfection with PMO-Peps induced high levels of exon 6-9 skipping at all time-points and by day 10, PMO-Peps were the only compounds to have sustained exon skipping (Fig 2.5D).

2.3.4 Dystrophin expression in treated cells

Western blot analysis was performed on protein extracted from AO transfected myotube cultures to confirm the proof of concept that the restoration of the in-frame dystrophin mRNA transcript would result in the production of dystrophin protein and provide a further comparison of the efficacy of the AO chemistries in restoring dystrophin production. Following 14 days of differentiation, myotubes cultures were transfected with either 20μM of PMO-Pep, 20μM PMO or 1μM 2OMe AO lipoplexes for both C6A(+69+91) and C8A(-04+18) AOs with protein and RNA extracted 7 days later. Western blot analysis using NCL-Dys1 demonstrated the low level induction of a shortened dystrophin protein in both PMO-Pep and PMO treated cultures, but not in the 2OMe AO transfected or the untreated cultures (Fig 2.6A). Detection of β-spectrin was used as a control for protein loading onto the Western gel. RNA analysis of treated cultures confirmed the induction of the in-frame transcript, albeit only at low levels in both PMO and 2OMe treated cultures (Fig 2.6B).
2.4 Discussion

The presence of dystrophin-positive revertant fibres in dystrophic tissue is evidence of a low level of endogenous exon skipping, whereby the disease-causing mutation is excluded. These revertant fibres occur at a frequency too low to have any clinical benefit. However, well documented cases of variably affected BMD patients, in some cases with major genomic deletions and very mild phenotypes, are evidence that the production of some shortened in-frame deleted dystrophin proteins can have a major benefit. To date, studies in the \textit{mdx} mouse and in DMD patient cell lines have demonstrated the use of AOs to manipulate pre-mRNA splicing to induce shortened in-frame transcripts to produce substantial amounts of dystrophin protein (Lu et al., 2003, Aartsma-Rus et al., 2003). In this study, we report the first use of AOs to induce \textit{in vitro} production of dystrophin in a canine model of DMD.

It has been our experience that one of the more crucial aspects of AO induced exon skipping is the selection of the target and design of AOs. The removal of exons from the pre-mRNA is presumed to be caused by steric interference by the AOs preventing the correct assembly of the spliceosome. Previous studies in the \textit{mdx} mouse indicated only AOs targeted to the 5’ donor splice site of exon 23 induced efficient and consistent skipping (Mann et al., 2001). The targeting of the 3’ splice site of intron 22 had no effect on the mouse dystrophin pre-mRNA splicing, while in the present study, targeting the 5’ donor splice sites did not induce skipping of either exon 6 or 8. Whilst the first series of AOs designed to the GRMD dystrophin pre-mRNA were targeting a crucial domain involved in splicing, it is possible that these AOs were not able to overcome secondary structures in the pre-mRNA and hence displace splicing factors. The MFold RNA folding program (http://www.bioinfo.rpi.edu/applications/mfold/) (Zuker, 2003) was used to predict areas of accessibility but these programs may be of limited value, as multiple RNA structures are predicted and will vary depending on the input of target sequence. In contrast to the amenable exon 23 donor splice site target in the \textit{mdx} model, targeting the 3’ acceptor splice site of canine dystrophin intron 7 was very effective in inducing exclusion of exon 8 from the mature mRNA. However, exon 8 removal was dependant on precise targeting where only one of two AOs directed to this splice site was able to induce exon skipping.
(Fig 2.3B). Unlike exon 8 removal, targeting of either acceptor or donor splice sites of exon 6 did not induce any detectable exon removal.

Studies in DMD patient cell lines demonstrated that targeting of putative exon splicing enhancers was able to induce skipping of targeted exons (van Deutekom et al., 2001). In silico prediction of ESEs has been developed to identify potential regions involved in human exon recognition and definition. One such program is ESE Finder (Cartegni et al., 2003) which predicts potential binding sites for serine/arginine rich (SR) proteins, a family of structurally related and highly conserved splicing factors, characterised by RNA recognition motifs (RRM) and an arginine/serine (RS) domain (Birney et al., 1993). The RRM domain confers exon specificity and recognition through binding to specific RNA sequences. The RS domain is thought to either promote exon definition by recruiting splicing factors and/or by inhibiting the actions of nearby silencer elements (Blencowe, 2000). In general, ESE Finder predicts potential ESEs within exons, with estimates based on homology to consensus sequences. This program identified several motifs within exon 6, one of which was targeted by C6A(+69+91) to induce skipping of that exon. It is assumed that this AO binding disrupted essential RRM base pairing with exon 6, thereby preventing binding of some SR proteins and retention of exon 6 in the mature mRNA. Interestingly, the AO C8A(-04+18) that spans the intron 7/exon 8 junction also targeted a region identified as binding potential ESEs. This may give an indication as to why the C8A(-10+10) AO could not induce skipping, as despite overlap in the targeted sequence, this AO may not displace enough of the SR protein binding to disrupt splicing. However, the exon 6 AOs targeted to the acceptor and donor sites which did not induce exon skipping, also targeted putative SR protein binding regions. Thus, it appears that whilst prediction of ESE sites may provide potential targets for AOs, empirical testing will still remain necessary as there is no obvious motif that will guarantee exon exclusion after AO targeting. Compounding the limitations of AO target prediction is that current ESE programs are modeled on human SR proteins, and so may be less relevant when designing AOs for other species. Even when predicting AO binding sites in the human dystrophin gene transcript, it is our experience that not all identified potential ESEs will induce exon skipping when targeted with AOs (unpublished data).
The non-specific exclusion of exon 9 was observed in about 50% of canine dystrophin transcripts from untreated cells and has also been observed in human and mouse muscle (Reiss and Rininsland, 1994). This constitutive skipping is thought to be due to the 3' end of exon 9 reflecting the consensus sequence of an intronic 3' splice site, such that it is 'recognised' as an intron and removed from the mRNA transcript (Reiss and Rininsland, 1994). As such, it was not surprising to observe frequent removal of exon 9 in dystrophin transcripts missing exons 6 or 8, or both. The removal of exon 9 from the mature mRNA will not disrupt the reading frame and hence should not have a detrimental effect on dystrophin expression. Perhaps reflecting the close co-ordination of exon 8 and 9 processing, the AO induced removal of exon 8 was always accompanied by the loss of exon 9, suggesting that the splicing of exon 8 and 9 are inextricably linked, whereas this was not the case for exon 6 skipping. The sizes of the introns involved may also influence splicing patterns, as intron 7 is approximately 97kb while intron 8 is only 1% of this length and intron 9 is over 25kb in length. This feature of a single AO removing two exons has also been observed in studies in the \textit{mdx} mouse, where exon 22 and 23 removal was frequently seen when AOs were targeted to exon 23 (Mann et al., 2002). Similarly, a high dose of AO targeted to exon 46 occasionally induced removal of both exon 45 and 46 from human dystrophin transcripts (Aartsma-Rus et al., 2004b).

Nested RT-PCR analysis was used to compare the efficacies of the different AO chemistries as the GRMD dystrophin mRNA level is too low to detect by Northern analysis (Sharp et al., 1992) and real-time PCR analysis is unsuitable for quantitation of multiple transcripts of varying size. Whilst nested RT-PCR does not allow for the precise calculation of the levels of exon skipping observed, the comparison in efficacy can be considered semi-quantitative as amplification conditions remained constant throughout the study. The apparent efficacy of exon skipping using 2OMe C6A(+69+91) and C8A(-04+18) at low lipoplex doses (to 20nM) was very high (Fig 2.4A). This multiple exon skipping is most remarkable when one considers that all four exons removed from the transcript, one by a splice site mutation and three through the AO action, span a considerable length of the pre-mRNA.

In contrast, although skipping of exons 6 and 8 was observed with the PMO of equivalent sequence, it was only observed after transfection at higher AO concentrations
As PMOs are uncharged, they cannot be complexed with cationic liposomes and presumably rely on passive diffusion through the cellular membrane. It has become apparent that this poor cellular uptake is the cause of low levels of exon skipping rather than an inability of the PMO to exert its antisense effect. In mdx mouse studies, the use of a sense "leash" with a charged phosphodiester or phosphorothioate backbone enabled the PMO to be complexed with liposome and induce levels of exon skipping at relatively low concentrations (~10nM) (Gebski et al., 2003). Although this greatly enhanced the PMO induced exon skipping, this complex preparation could be difficult to apply in a clinical setting.

The conjugation of a transport peptide facilitated nuclear uptake, with high levels of induced in-frame transcript demonstrating that when the PMO can be effectively transported into the cell, it exerts potent and sustained antisense activity. Additionally, in terms of a clinical application, it would be preferable to use a chemistry that did not require a transfection agent, since a limitation of using cationic liposomes as a carrier is associated toxicity and aggregation with serum proteins in vivo (Wu et al., 2001). This was demonstrated by moderate cell death observed 7 days after transfection with 600nM of 2OMe AO lipoplex that limited the use of higher doses of 2OMe AOs. The absence of any obvious adverse affects on the adherent cells by the PMO and PMO-Pep AOs at 20μM suggests even higher doses may be employed in vitro.

A study of the persistence of the in-frame transcript missing exons 6-9 indicated that whilst the 2OMe AOs were initially very effective, the shortened transcript declined rapidly. Whilst there was an observed increase in cell death over time, this toxicity was not enough to account for the decline in induced transcript and is most likely due to metabolism and decay of the 2OMe AO. This pattern of induced exon skipping is similar to that reported in the mdx mouse, where shortened transcripts were observed on day 1 and only low amounts were present 10 days following in vitro AO transfection (Mann et al., 2002). However, there was still some skipping of either exon 6 or 8 alone at day 10, suggesting that some 2OMe AO must still be present, as these out-of-frame transcripts should be rapidly turned over due to nonsense mediated decay. Given that the PMOs are taken up poorly in vitro, it was not surprising that almost negligible exon skipping was observed for all time-points. However, as uncomplexed PMOs rely on passive diffusion to enter the cell,
it was hypothesized that the level of induced exon skipping would increase over time, though this was not the case. This was in stark contrast to the PMO-Pep, where at the same PMO concentration, the use of a conjugated transport peptide overcame limitations in the *in vitro* delivery to induce high levels of skipping at all time-points. This suggests that once the PMO is in the cell, it remains more stable than the 20Me chemistry and hence can exert more sustained antisense activity, although this activity may be enhanced by an absence of toxicity. Additionally, use of a PMO:leash lipoplex in the *mdx* mouse resulted in increased persistence of exon 23 removal in comparison to 20Me AO lipoplex, indicating that the PMO chemistry appears to exert its antisense effect for longer (Gebski et al., 2003). The ability of the PMO-Pep to be delivered as a simple formulation also reduces potential problems associated with carrier agents and could simplify clinical application. Thus, despite the 20Me AO chemistry being able to induce exon skipping at a lower concentration for a short period (20nM) compared to the PMO-Pep (300nM), the lack of adverse effects of the PMO allows higher doses to be used, and hence induce maximal, sustained exon skipping.

One limitation of using GRMD primary myoblasts is the poor ability of the cells to differentiate in cell culture. This in turn presents difficulties in studying dystrophin protein, which is expressed late in the differentiation process and accounts for only 0.002% of total muscle protein. Despite this, near full-length dystrophin protein could be detected by Western blot using high dose transfection of uncomplexed PMO and PMO-Pep (Figure 2.6A). A direct comparison of AO chemistries at the same concentration could not be made, as 20Me AO lipoplex concentrations in excess of 1μM induced excessive cell death. It was somewhat surprising that the PMOs alone were able to induce any detectable dystrophin protein, however the level of protein present is barely detectable. The level of exon 6 and 8 skipping at the time of protein extraction was very low but it seems likely that exposure of the PMO over time allowed some shortened in-frame transcript to accumulate and be translated (Fig 2.6B). Although the 20Me AO lipoplex treated cells also showed similar levels of exon skipping at the time protein was harvested, there were insufficient cells expressing dystrophin to allow detection by Western blotting. The PMO-Peps transfect efficiently at all stages of cell culture and maintained a high level of induced in-frame transcripts over time. Given that the total protein in the treated cultures was higher than in normal control protein, as determined by β-spectrin analysis, the level of induced
dystrophin is significantly lower. This was not unexpected as the relative level of dystrophin mRNA in GRMD compared to normal cell lines is vastly different. A comparison between GRMD and normal canine dystrophin mRNA levels indicated that GRMD mRNA was too low to detect by Northern analysis (Cooper et al., 1988, Sharp et al., 1992), presumably due to the rapid nonsense mediated decay of the out of frame GRMD transcript. Thus, whilst in normal cell lines all of the dystrophin transcripts have the potential to be translated into protein, only those GRMD transcripts that are corrected by AO mediated intervention will have the potential to be translated into an internally deleted dystrophin protein. Coupled with the difficulties in using GRMD cultures in vitro, this may contribute to the low level of induced dystrophin protein in comparison to normal dystrophin levels. Most importantly, the proof of concept that dystrophin protein can be induced in the GRMD model using this AO based approach, facilitates the testing of AOs in a highly relevant animal model of DMD. Future application to the canine model in vivo will provide information about the AO treatment regimens necessary to induce a functional benefit in a larger animal, as well as demonstrate any potential adverse effects. This in turn should give a better indication of the potential for success of this AO approach in human clinical trials as well as provide a platform for the testing of new AO chemistries and delivery techniques as they become available.

The disease progression and clinical similarities of the GRMD model to the human disease, as well as the larger size, makes affected dogs excellent models for preliminary studies prior to clinical trials. In addition, the location of the canine gene lesion involves an exon involved in the minor deletion hotspot in the human dystrophin gene. Removal of exon 8 alone would restore the reading frame of approximately 4.5% of DMD-deletion patients (van Deutekom and van Ommen, 2003). In-frame deletions in this region would be expected to produce a functional dystrophin protein, as evidenced by a report describing a very mild Becker muscular dystrophy patient with a 3-9 deletion, who was only diagnosed at the age of 65 years (Heald et al., 1994).

This work is a proof-of-principle study to develop AOs that could overcome the GRMD mutation and induce dystrophin expression. While 20Me AOs were more efficient at lower concentrations in inducing the in-frame dystrophin transcript, its limitations include the use of a delivery agent and the rapid decline in exon skipping levels over time,
suggesting that this chemistry may not be ideal. In comparison, uncomplexed PMOs could be used at much higher doses, and in doing so, have been shown to be able to induce dystrophin protein in vitro in the GRMD model. The use of a conjugated transport peptide overcame the problem of poor transfection efficiency of PMOs and subsequently showed that PMO-Peps could induce sustained and significant levels of exon skipping. We propose that PMOs and in particular PMO-Peps may be the most suitable chemistry available for the manipulation of exon splicing to address dystrophin mutations.

2.5 References


Figure 2.1 Structure of 2'-O-methyl phosphorothioate (2OMe), phosphorodiamidate morpholino oligomers (PMO) and peptide linked PMO (PMO-Pep). The structure of the conjugate is (RXR)$_n$XB where R=arginine, X=6-aminohexanoic acid and B=beta-alanine.
**Figure 2.2** Position of target AO sequences relative to the intron exon boundary of (a) exon 6 and (b) exon 8 of the GRMD dystrophin gene. * indicates AO sequences that induce exon skipping.
Figure 2.3 (a) Overview of exon skipping approach. GRMD mutation causes exclusion of exon 7 from the mRNA producing an out-of-frame transcript. AO targeted skipping of exon 6 and 8 induces splicing of exon 5 to 9 or 5-10, both of which result in an in-frame transcript. (b) Removal of exon 6 (173bp) and exon 8 (185bp) could only be induced by C6A(+69+91) and C8A(-04+18) respectively. (c) PCR amplification from exon 1-9 showing loss of exon 8 skipping, indicating that 8 removal is always accompanied by exon 9 skipping. Exon 6 skipping is independent of exon 9 as can be detected in amplification from exons 1-9 and 1-10. Titration of (d) C6A(+69+91) and (e) C8A(-04+18) from 600-20nM. Constitutive skipping of exon 9 is also shown to be present in all samples. △ indicates skipped exons.
Figure 2.4 Comparison of AO chemistries to induce removal of exon 6 and 8 from mRNA. The 466bp product indicates corrected in-frame transcript splicing exon 5 to exon 10. Titration of (a) 2OMe, (b) PMO and (c) PMO-Pep AOs to induce exon 6 and 8 skipping. (d) Sequencing of 466bp product indicating precise splicing of exon 5 to exon 10. Δ indicates skipped exons.
Figure 2.5 RTPCR analysis of time course of induction of in-frame transcript (466bp) by 2OMe, PMO and PMO-Pep AOs (a) 1 day, (b) 4 days, (c) 7 days and (d) 10 days following a single dose transfection. Some heteroduplex formation can be observed in (a) above the 466bp product. Δ indicates skipped exons.
Figure 2.6 (a) Western blot for dystrophin muscle isoform (427kDa) in PMO-Pep, PMO and 2OMe treated cells by western blot. Lower band in PMO-Pep and PMO treated cells indicates the expected shortened dystrophin product. B-spectrin (235kDa) was used as a control for protein loading. Normal control was taken from unaffected GRMD littermate cultures. (b) RT-PCR analysis of treated cell cultures used in protein analysis. Δ indicates skipped exons.
Chapter 3

Production of dystrophin *in vivo* in a canine model of DMD

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3.1 Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder caused by an absence of functional dystrophin that results in progressive muscle degeneration, with death typically caused by respiratory or cardiac failure. Currently there is no effective treatment for this disease and with approximately one third of mutations *de novo* (Laing, 1993), it cannot be eliminated through genetic counseling. Whilst corticosteroid supplementation (Alman, 2005) and assisted ventilation (Mellies et al., 2003) can prolong and improve life, they do not address the primary genetic defect and a number of gene therapy strategies are undergoing investigation. Animal models of disease play an integral role in this process and for DMD, the majority of preclinical studies have focused on the use of the *mdx* mouse. A nonsense mutation in exon 23 of the dystrophin gene precludes the synthesis of a functional protein (Sicinski et al., 1989); however the animal remains relatively clinically normal until later in life. In contrast, the canine model of DMD has striking phenotypic similarities to the human disease and are closer in size, an important factor for consideration of therapeutic delivery. In golden retriever muscular dystrophy (GRMD), characteristics of disease progression include severe skeletal myopathy with contractures, elevated serum creatine kinase levels, fiber degeneration and regeneration, fiber mineralization and premature death (Valentine et al., 1990). The GRMD model also displays the presence of rare dystrophin positive “revertant” fibers which are also present in
the mouse model and in human disease (Schatzberg et al., 1998). Whilst these features make it an excellent clinical model of DMD, it has not been widely used in the field of DMD research, predominantly due to the high maintenance cost and relative scarcity of breeding colonies. The availability of animals for study is also complicated by a longer gestation period and smaller litters than for the mouse model. Despite these limitations, as success in various therapeutic approaches in the mdx mouse provide promise for application in humans, the importance of using a more clinically relevant model prior to clinical trials is evident.

The genetic basis for the GRMD model is a point mutation in the acceptor splice site of intron 6 of the dystrophin gene that affects recognition of exon 7, such that it fails to be spliced into the mature mRNA transcript (Sharp et al., 1992). Deletion of exon 7 from the dystrophin mRNA causes a frame-shift that subsequently induces a stop codon in exon 8, resulting in the loss of a functional protein (Sharp et al., 1992). Whilst splice site mutations are relatively rare in the human disorder (~7%), therapeutic approaches to overcome this mutation are highly relevant, as genomic exon deletions that result in a mRNA frame-shift are the most common type of mutation observed in DMD (Roberts et al., 1994). Genetic therapies in the GRMD model to date have focused on a number of approaches. Gene replacement of either full-length or mini- or micro-dystrophin cDNA has been attempted by local delivery of either plasmid (Howell et al., 1997), adenoviral (Howell et al., 1998), or recombinant adeno-associated viral (rAAV) vectors (Wang et al., 2007b), with variable dystrophin positive expression tempered by issues related to immune response to the viral vectors. Chimeric RNA/DNA oligonucleotides have been used for targeted gene repair of the intron 6 acceptor splice site, such that host cell mismatch repair would induce permanent correction of the chromosomal mutation (Bartlett et al., 2000). Although low levels of dystrophin protein were detected, expression was sporadic and even in sites of highest dystrophin expression only 6% of PCR clones derived from treated muscle exhibited correction of the point mutation (Bartlett et al., 2000). Upregulation of the functionally homologous utrophin, a foetal form of dystrophin, has been used to attempt to compensate for the absence of dystrophin. Adenoviral vector expression of a mini-utrophin cassette was stable for at least 60 days, resulting in reduced fibrosis and increased expression of dystrophin-associated proteins (Cerletti et al., 2003). However, there does not seem to be any significant advantage of upregulation of a homologous protein when a
functional dystrophin can be delivered using similar vector systems. Cell replacement therapy has also been investigated in the GRMD model by intra-arterial delivery of wild-type canine mesoangioblasts (vessel-associated stem cells) (Sampaolesi et al., 2006). Dystrophin expression in various muscles was variable but large areas of dystrophin-positive fibres could be detected with associated preservation of morphology. Functional improvement was also observed with increased tetanic force of contraction and evidence of improvement of mobility of some animals (Sampaolesi et al., 2006). Although this approach offers exciting prospects; immune suppression is a requirement for cell transplantation and it was interesting to note that in this study, cessation of immune suppression resulted in half of the dogs that demonstrated a clinical improvement, rapidly losing walking ability (Sampaolesi et al., 2006).

A recent therapeutic approach to address DMD mutations has been the use of antisense oligonucleotides (AOs) to manipulate pre-mRNA splicing to restore the mRNA reading frame (Mann et al., 2001, McClorey et al., 2006, van Deutekom et al., 2001). Whilst AOs have been traditionally used to down-regulate mRNA transcripts though RNase-H mediated degradation, in this approach, AOs are applied to bind to specific splicing motifs to prevent recognition of the targeted exons by the cellular splicing machinery. For the GRMD model, targeted removal of exons 6 and 8 from the dystrophin transcript would restore the mRNA reading frame to allow synthesis of a protein of reduced function. Internally deleted dystrophin protein is known to have some functionality, as evidenced by patients with the allelic Becker muscular dystrophy. Depending on the size and position of this deletion, patients have phenotypes ranging from borderline DMD to almost asymptomatic (Beggs et al., 1991, Ringel et al., 1977). Thus it would be anticipated that application of this approach to amenable dystrophin mutations should be beneficial. This approach has been used successfully in the \( mdx \) mouse model, where systemic restoration of dystrophin expression has showed functional improvement in muscle strength testing and reduction in numbers of regenerating fibres (Alter et al., 2006, Fletcher et al., 2007, Fletcher et al., 2006, Lu et al., 2003). In the GRMD model, the principle of this approach has been shown in vitro in GRMD derived myocytes, with correction of the dystrophin mRNA and subsequent induction of protein reported (McClorey et al., 2006). In this study, we present data from pilot in vivo studies of local administration of previously reported AOs to induce dystrophin expression in affected GRMD animals.
3.2 Materials and Methods

3.2.1 Animals

Dogs were obtained from the GRMD colony held at the University of Missouri-Columbia and cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Identification of affected dogs was based on marked elevation of serum creatine kinase and confirmed by PCR-based genotyping (Sharp et al., 1992).

3.2.2 Treatment regimen

AOs were transported to USA and injections performed at University of Missouri-Columbia. For each treatment regimen a single affected animal was administered with AOs targeting exon 6 and 8 for excision from the canine dystrophin transcript (Table 3.1). AO sequences were based on previous in vitro studies with 2'-O-methyl phosphorothioate (2OMe), phosphorodiamidate morpholino oligomer (PMO) and peptide-linked PMO (PMO-Pep) tested (McClorey et al., 2006). For the 2OMe regimen, AOs were complexed with either lipofectin (Invitrogen, Victoria) at a 2:1 wt:wt ratio according to manufacturer’s instructions; or the nonionic block copolymer F127 as described (Lu et al., 2005), prior to injection. Both the PMO and PMO-Pep AOs were injected uncomplexed in a physiological saline solution. Contralateral injections were made into the lateral digital extensor with the needle inserted longitudinally, and muscle removed 1 or 3 weeks post injection for histological analysis.

3.2.3 Histology

Muscles were removed under general anesthesia using open surgical technique as described previously (Childers et al., 2001). Tissue was snap frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Serial lateral sections (6μM) were cut through the whole muscle using a Leica Jung CM1800 cryostat (Bremen, Germany). Immunohistochemistry was performed on unfixed frozen sections using a mouse monoclonal antibody NCL-DYS1 (Novocastra Laboratories, Newcastle, UK), that reacts strongly with the dystrophin rod domain. Signal was detected using a biotinylated anti-
mouse secondary antibody in conjunction with an avidin-biotin-peroxidase Vectastain Elite ABC Kit (Vector Laboratories, CA, USA) and visualised with diaminobenzidine (DAB) substrate (Vector Laboratories, CA, USA).

3.3 Results

3.3.1 2OMe treatment

Tissues from age-matched, unaffected littermates were used as a positive control for the dystrophin staining procedure (Figure 3.1a). Dystrophin-positive staining is indicated by strong, consistent brown staining localized to the membrane of the muscle fibres. Staining of sections from age-matched, untreated affected littermates was used as a negative control, with negligible signal observed above background DAB staining (Figure 3.1e). Typical features of dystrophic muscle can also be observed, such as non-uniform fibre size due to muscle degeneration and regeneration, and fibrotic infiltration between fibres. Analysis of serial sections of muscle injected with 25μg each of 2OMe AO6 and AO8 complexed with lipofectin, revealed almost no positive fibres throughout the tissue at both 1 week (Figure 3.1 b, c, d) and 3 weeks (Figure 3.1 f, g) post injection. Any observed dystrophin-positive fibres were located towards the edge of muscle bundles bordering connective tissue, and had a discontinuous staining pattern. Similarly, for the muscles injected with 2OMe AO6 and AO8 with F127, only a very small percentage of fibres exhibited dystrophin-positive staining after 1 (Figure 3.2 b, c, d) and 3 weeks (Figure 3.2 f, g). These fibres were also located at the periphery of muscle fascicles and in close proximity to blood vessels or the needle injection tract.

3.3.2 PMO treatment

Age matched normal and affected littermates were used for dystrophin-positive (Figure 3.3a) and dystrophin-negative (Figure 3.3e) controls respectively. As the production of dystrophin was almost negligible in 2OMe AO treated muscles, the dosage of PMO AO6 and AO8 was increased such that 75μg of each AO was administered. Injection of the PMO AO6 and AO8 resulted in the induction of dystrophin-positive fibres along the length of the tissue, but particularly in the longitudinally central sections of the muscle. A heterogeneous staining pattern could be observed at both the periphery and within muscle
fascicles, with positive fibres easily discernable from neighboring dystrophin-negative fibres (Figure 3.3). Dystrophin was localized to the membrane, with some fibres displaying a continuous staining whilst others exhibited only partial dystrophin staining (Figure 3.3d). After 3 weeks, dystrophin positive fibres could still be detected, although staining was not as widespread throughout the whole muscle. In areas of dystrophin expression, the numbers of positive fibres were reduced and appeared to be confined mainly to the edge of fascicles.

3.3.3 PMO-Pep treatment

Peptide-linked PMO AOs were injected into a 1 week-old affected pup to determine firstly; if the efficacy of the PMO chemistry could be improved by addition of a cell penetrating peptide, and secondly if this efficacy was increased in a younger animal. The amount of PMO-Pep AO injected was increased to 175μg of each AO in an attempt to improve dystrophin expression levels over that observed with the PMO chemistry. Unfortunately during surgery, the lateral digital extensor muscle analysed 1 week post injection, was damaged due to complications in locating it within the leg, whereas the 3 weeks contralateral muscle was injected without difficulty. In the muscle sample analysed after 1 week, significant numbers of fibres were lost presumably due to tissue damage, with fibrotic infiltration throughout the muscle, that makes interpretation of results difficult (Figure 3.4b). However, of the remaining fibres, the majority show high levels of dystrophin-expression. In the contralateral muscle removed 3 weeks post injection, widespread heterogeneous dystrophin staining was observed throughout the length of the muscle, with significant spread through the fascicles (Figure 3.4 c, d, f and g). Patterns of dystrophin localization varied between fibres; with the majority exhibiting only partial staining at the membrane and of a lower intensity in comparison to the PMO treated muscles.

3.4 Discussion

The principle of using AOs to correct the splicing defect in the GRMD model has been previously demonstrated in vitro (McClorey et al., 2006). Targeted excision of exons 6 and 8 converted an out-of-frame dystrophin transcript to an internally deleted but in-frame transcript that allowed synthesis of near full length dystrophin protein. Whilst a
cellular model permits optimisation of this approach, the use of a monolayer myocyte culture is not an accurate representation of the complexity of the skeleto-muscular system. To validate this approach further, intramuscular injection of 2OMe, PMO and PMO-Pep AO analogues was performed. One of the major limitations of the use of the GRMD model in this study was the availability of animals. Unlike the *mdx* mouse which can be bred quickly and in large numbers, the high cost and difficulty in breeding of the GRMD animal meant that only limited studies could be performed. Whilst this precludes statistical analysis of the data, it was sufficient to demonstrate the proof, or lack thereof, of this approach.

Injection of 2OMe AOs induced almost negligible dystrophin staining in muscle after 1 or 3 weeks, for both lipofectin and F127 complexed AOs (Figure 3.1). Although some dystrophin-positive fibres were observed, they were infrequent throughout the tissue and it could be argued that they were naturally occurring "revertant" fibres that are found at a frequency of less than 1% (Kornegay et al., 1994). This was surprising, given the precedent of lipofectin-conjugated 2OMe AOs being used for the initial demonstration of exon skipping for *in vivo* correction of the dystrophin mutation in the *mdx* mouse model (Mann et al., 2001). Similarly, F127 had been used as a carrier for 2OMe AOs to demonstrate considerable improvement of dystrophin expression in the *mdx* mouse *in vivo* (Lu et al., 2003). Use of 2OMe AOs in the GRMD *in vitro* model induced efficient correction at the RNA level in myoblasts but was not able to induce dystrophin protein in myocytes (McClorey et al., 2006). This is reflected in the *in vivo* results and is possibly due to a reduced efficacy of this chemistry when directed against the more mature muscle fibre. Although the AO concentration used in this study (50μg total) is comparative to the *mdx* studies (5μg) when the size of the injected muscle is considered, it is conceivable that an increased dose would improve dystrophin expression. However, one of the limitations of using 2OMe AOs is the toxicity associated with the use of a carrier to transport the negatively charged nucleic acid across the cell membrane that would preclude the use of very high doses. There is also the potential for the phosphorothioate backbone to induce a non-specific immune response through activation of CpG motifs and Toll-like receptors, similar to that observed for short interfering RNA molecules (Agrawal and Kandimalla, 2004) although this remains to be extensively tested. Given that the 2OMe AOs worked
very poorly by local injection, they are unlikely to have any beneficial effect through systemic delivery, at least in the GRMD model.

In contrast, both of the PMOs induced a widespread pattern of dystrophin expression throughout the injected muscle. The localisation of staining to the sarcolemma indicates that a near full length, partially functional protein is being induced, as the C-terminal cysteine-rich region is necessary for binding to β-dystroglycan (Lenk et al., 1996). The discontinuous pattern of staining that is observed around some fibres is similar to the patchy staining seen in some Becker muscular dystrophy patients (Morandi et al., 1995). This is not surprising, given that the therapeutic approach is mimicking the internally deleted in-frame transcripts that characterize the milder allelic BMD. For both PMO and PMO-Pep AOs, dystrophin-positive fibres can be found deep within muscle fascicles, suggestive of a capability of these AOs to spread beyond the injection tract; although there do appear to be increased numbers of positive fibres adjacent to connective tissue. This pattern is likely to be due to a gradient of AO availability, as the majority of the AO would be expected to be taken up by fibres in closest proximity to the needle tract or circulatory system. In this case, it would suggest that relatively low levels of AO are required to produce detectable amounts of dystrophin in fibres deep within fascicles. It may also help explain the observed pattern of strongly positive fibres adjacent to negative fibres. If high concentrations of AOs were necessary to induce dystrophin expression then it would be expected that these strongly staining dystrophin-positive fibres would by surrounded by fibres of equal intensity or less, but not totally devoid of signal.

The efficacy of the PMO chemistry was not predicted by in vitro studies where high concentrations of uncomplexed PMO did not induce dystrophin protein and had resulted in only low levels of correction at the RNA level (McClorey et al., 2006). This correlates well with studies in the mdx mouse model where the use of a phosphodiester “leash” was necessary to facilitate the use of cationic liposomes to enhance uptake of PMO AOs in vitro (Gebski et al., 2003). However, systemic delivery of uncomplexed PMO AOs in vivo induced sustained and widespread dystrophin expression throughout the animal (Alter et al., 2006, Fletcher et al., 2006). The precise mechanism of uptake of the PMO chemistry is unknown, but it could be surmised that it is through passive diffusion due to its non-ionic nature. However, this does not explain the widely contrasting efficacy observed for in vivo
and *in vitro* systems. Higher concentrations of PMO were used as a result of the poor efficacy shown by the 2OMe AOs and the expected low efficacy based on *in vitro* results. As such, a direct comparison between these two chemistries with identical concentrations was not possible for this study, however evidence would suggest that both the PMO and PMO-pep chemistries have a higher efficacy. In comparing the PMO and PMO-Pep chemistries, it was expected that the numbers of dystrophin-positive fibres would be substantially higher in the PMO-pep treated animal given that only PMO-pep compounds had sufficient efficacy to induce dystrophin expression *in vitro*, presumably as the conjugated cell penetrating peptide facilitated its uptake into the cell nucleus (McClorey et al., 2006). Additionally, in the experimental design, an increased concentration of AO was used in a younger animal, and hence a smaller muscle, in an attempt to maximize the induction of dystrophin. That increased numbers of dystrophin-positive fibres were not observed, and dystrophin staining was not as pronounced as for PMO treated muscle, suggests that the conjugated peptide did not confer any advantage *in vivo*. However, this data is based on a single animal data and further studies would be necessary to determine if this was a trend or an anomaly.

Relatively few studies have demonstrated restoration of dystrophin expression *in vivo* in the GRMD model and to date, there have been no published reports utilizing an exon skipping approach. Injection of either a full-length or a shortened “mini-dystrophin” cDNA plasmid was shown to induce less than 1% of fibres to express dystrophin and even then only around the site of injection (Howell et al., 1997). This was improved through the use of an adenoviral vector to deliver a human mini-dystrophin where up to 50% of fibres expressed dystrophin around the site of intramuscular injection (Howell et al., 1998). However humoral and cellular immune responses to antigens of both viral and transgene origin, lead to a decline of dystrophin expression over a two month period (Howell et al., 1998). This highlights an important consideration for the use of viral vectors, namely, an elicited immune response to the delivery vector itself. rAAV-based vectors are thought to have great potential to deliver genes to muscle cells, due to their ability to transduce replicating as well as non-replicating cells (Athanasopoulos et al., 2000), and the affinity that some serotypes have for striated muscles (Blankinship et al., 2004, Wang et al., 2005). Intramuscular injection into random-bred dogs with rAAV vectors carrying various transgene cassettes elicited a robust primary cellular immune response, regardless of the
transgene expressed, cellular specificity of the promoter, or the muscle type injected (Wang et al., 2007a). This was in direct contrast to studies in the mdx model where delivery of a micro-dystrophin cassette using the same viral vector did not induce an immune response (Liu et al., 2005). To address the immunogenicity, a brief course of immunosuppression was used to augment the delivery of a canine micro-dystrophin transgene using rAAV vectors intramuscularly (Wang et al., 2007b). This regimen suppressed the immune response to the vector and allowed robust and prolonged expression of the canine micro-dystrophin for up to 3 months after withdrawal of immunosuppression. Whilst these results are promising, it is likely that repeat viral transductions of a dystrophin transgene would be necessary for successful clinical treatment and this would be limited by the ability to suppress the immune response. Although the level of dystrophin expression using PMO AOs was not as robust as for the rAAV micro-dystrophin vectors, an immune response to nucleic acid would not be predicted and indeed PMO AOs have been used in Phase I/II clinical trials with no adverse effects reported in over 250 treated patients (http://www.avibio.com/devNeugene.html). The absence of adverse effects would therefore allow for re-administration of PMOs to enhance dystrophin expression to levels that would have functional benefit.

The use of AO mediated exon skipping to restore dystrophin expression in the GRMD model has been demonstrated for the first time in this study. Although data has been limited by the availability of animals, the proof of principle of this approach in a more clinically relevant animal model provides an excellent platform for which to explore optimal dosing and delivery regimens, prior to clinical application in humans. The poor result obtained using the 2OMe chemistry, suggests that it would be unsuitable for further in vivo investigation. However, it will be necessary to extend the morpholino AO based studies to confirm the reproducibility of these results as well as ascertain the optimal delivery regimen to attain the highest level of dystrophin expression. Finally, systemic delivery of the morpholino AOs in an animal model closer to human size will lead to a clearer indication of the distribution and stability of dystrophin expression and the functional improvement that could be anticipated from their use in clinical trials.
3.5 References


<table>
<thead>
<tr>
<th>Animal</th>
<th>Age</th>
<th>Muscle injected</th>
<th>Injected material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4mo</td>
<td><em>Lateral digital extensor</em></td>
<td>50μg of 2’-OMe AO 6 + 2’-OMe AO 8 with lipofectin</td>
</tr>
<tr>
<td>2</td>
<td>5mo</td>
<td><em>Lateral digital extensor</em></td>
<td>50μg of 2’-OMe AO 6 + 2’-OMe AO 8 with F127</td>
</tr>
<tr>
<td>3</td>
<td>3mo</td>
<td><em>Lateral digital extensor</em></td>
<td>150μg of PMO AO 6 + PMO AO 8</td>
</tr>
<tr>
<td>4</td>
<td>1w</td>
<td><em>Lateral digital extensor</em></td>
<td>350μg of PMO-Pep AO 6 + PMO-Pep AO 8</td>
</tr>
</tbody>
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**Table 3.1** Summary of treatment regimens for GRMD animals used in this study.

Contralateral injections were administered into the *lateral digital extensor* and removed 1 and 3 weeks later. AO sequences were as described in Figure 2.2
3.6 Figures

**Figure 3.1 2OMe AO with lipofectin carrier.**
Dystrophin staining of 6µM transverse sections from \textit{lateral digital extensor} muscle. Age matched controls of (a) normal and (e) affected littermates. Dystrophin expression after treatment with 50µg of 2OMe AO 6 and AO 8 with lipofectin at various sites after 1 week (b, c) 200x and (d) 400x magnification; and 3 week (f) 200x and (g) 400x, post-injection. Scale bar = 200µM. Arrows indicate areas of dystrophin-positive fibres.

**Figure 3.2 2OMe AO with F127 carrier.**
Dystrophin staining of 6µM transverse sections from \textit{lateral digital extensor} muscle. Age matched controls of (a) normal and (e) affected littermates. Dystrophin expression after treatment with 50µg of 2OMe AO 6 and AO 8 with F127 at various sites after 1 week (b, c) 200x and (d) 400x magnification; and 3 week (f) 200x and (g) 400x, post-injection. Scale bar = 200µM. Arrows indicate areas of dystrophin-positive fibres.

**Figure 3.3 Non - complexed PMO AO.**
Dystrophin staining of 6µM transverse sections from \textit{lateral digital extensor} muscle. Age matched controls of (a) normal and (e) affected littermates. Dystrophin expression after treatment with 150µg of PMO AO 6 and AO 8 at various sites after 1 week (b, c) 200x and (d) 400x magnification; and 3 week (f, g) 200x and (h) 400x, post-injection. Scale bar = 200µM. Arrows indicate areas of dystrophin-positive fibres.

**Figure 3.4 PMO-Pep AO.**
Dystrophin staining of 6µM transverse sections from \textit{lateral digital extensor} muscle. Age matched controls of (a) normal and (e) affected littermates. Dystrophin expression after treatment with 350µg of PMO-Pep AO 6 and AO 8 at various sites after 1 week (b) 200x; and 3 week (c, f) 200x and (d, g) 400x, post-injection. Scale bar = 200µM. Arrows indicate areas of dystrophin-positive fibres.
Figure 3.1 20Me AO with lipofectin carrier
Figure 3.2 2OMe AO with F127 carrier
Figure 3.3 Non-complexed PMO AO
Figure 3.4 PMO-Pep AO
Chapter 4

Multiple dystrophin exon skipping to induce a gene transcript found in a phenotypically mild Becker muscular dystrophy

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4.1 Introduction

The absence of a functional dystrophin protein is the cause of Duchenne muscular dystrophy (DMD), a severe muscle wasting disease that typically results in death by the third decade (Emery, 2002). Dystrophin contributes to muscle stability during contraction by providing a structural link between the cytoskeleton and basal lamina of the extracellular matrix (Michalak and Opas, 1997, Rando, 2001). Complete loss of this protein results in cycles of muscle fibre degeneration/regeneration that underlie the severe DMD pathology. In contrast, the allelic disorder Becker muscular dystrophy (BMD) has a spectrum of phenotypes, ranging from very mild to borderline DMD (Beggs et al., 1991, Ringel et al., 1977). This variation in severity and disease progression is due to the nature and position of the patient mutation. Generally, an in-frame deletion in the dystrophin gene induces an internally deleted protein that retains some functionality (Koenig et al., 1989, Gillard et al., 1989). In-frame deletions in the distal rod domain account for the majority of “classical” BMD cases who may remain ambulant well past their twenties (Beggs et al., 1991). Indeed, a deletion of exons 17 to 48, which encompasses 46% of the dystrophin coding sequence, was found in a patient who remained ambulant at 61 years of age (England et al., 1990). In contrast, many BMD mutations involving the amino terminus have low protein levels, early onset and more severe progression (Beggs et al., 1991), although there are exceptions such as a patient with an exon 9-22 deletion that had high creatine kinase levels but well developed musculature and an absence of muscle weakness (Gospe et al., 1989). Interestingly, the frame-shift deletion of exon 3-7 results in BMD, DMD or an intermediate phenotype (Malhotra et al., 1988). This variation is commonly thought to be due to
alternative splicing caused by natural exon skipping that allows patients to produce some functional dystrophin (Chelly et al., 1991).

As well as the semi-functional dystrophin proteins found in BMD, dystrophin-positive fibres can be found in more than 50% of DMD patients and most animal models of dystrophin mutations (Hoffman et al., 1990, Schatzberg et al., 1998, Fanin et al., 1992). These so called “revertant fibres” are the result of naturally occurring exon skipping that bypasses the DMD gene lesion, such that an internally deleted protein can be translated. The presence of these revertant fibres and the known reduction in phenotypic severity in BMD patients, led to the premise that efficient induced exon skipping could potentially rescue some functional protein expression from a dystrophin gene that carried a protein truncating mutation. In recent years, the potential of this approach has been demonstrated by use of antisense oligonucleotides (AOs) to modulate pre-mRNA splicing and excise exons to induce an in-frame dystrophin transcript (Aartsma-Rus et al., 2003, Lu et al., 2005, McClorey et al., 2006b). This in turn should result in the translation of an internally deleted but semi-functional dystrophin protein.

In vitro human studies to date have generally used AOs to excise single or multiple exons from the dystrophin transcript to induce the minimal change necessary to correct the reading frame and restore dystrophin production in DMD patient cell lines (Aartsma-Rus et al., 2004, Aartsma-Rus et al., 2003). It may however, be more advantageous to induce excision of several exons from the dystrophin transcript to produce a larger, in-frame deletion known to be associated with a mild BMD phenotype, thereby producing a potentially more functional dystrophin, compared to that induced by a minimal change. In this way, a single cocktail of AOs could address a range of DMD mutations within the scope of the in-frame deleted region, assuming that the primary gene lesion did not compromise any AO annealing site. A recent study of multiple exon skipping in the human dystrophin transcript, used either individual AOs or linked bi-functional AOs to target the removal of the outermost exons of multi-exon regions that would be applicable to 55% of all DMD patients (Aartsma-Rus et al., 2006). These experiments were largely unsuccessful, with no skipping of the targeted 17-48, 17-51 and 48-59 exons observed (Aartsma-Rus et al., 2006). However, multiple exon skipping was observed at a low
frequency when targeting exons 42-55 (4%) and exons 45-59 (6%), indicating for these regions at least, the proof of principle of this approach (Aartsma-Rus et al., 2006).

We hypothesized that it should be possible to induce removal of multiple exons by targeting each consecutive individual exon, as previously shown in studies in the mdx mouse (Fall et al., 2006). Targeting exons 19-25 of the mouse dystrophin using a cocktail of nine individual AOs, induced the splicing of exons 18-26, both in vitro and in vivo (Fall et al., 2006). In the current study, we induced the removal of exons 3-9 from the dystrophin transcript. This deletion was reported in a patient who was unaware of any impairment until age 65 and at the time of diagnosis at 67 years, had focal wasting of the quadriceps muscles but no pain, muscle cramps or fasciculation (Heald et al., 1994). Deletion of dystrophin exons 3-9 could be under-reported, given the extremely mild nature of the phenotype in this individual. A review of the Leiden BMD/DMD database (www.dmd.nl) indicates that generation of an exon 3-9 deletion could potentially be of benefit to approximately 7.5% of all DMD patients. As multiple exons require targeting, data presented here should also provide insight into the limitations of an AO induced exon skipping approach to mimic mild BMD mutations with large in-frame deletions.

4.2 Materials and Methods

4.2.1 Cell culture and AO transfection

Human muscle biopsies were obtained after informed consent from normal individuals undergoing elective surgery at Royal Perth Hospital, Western Australia. This project was approved by the University of Western Australia Human Ethics Committee (approval number RA/4/1/0962). Primary myoblasts were isolated following enzymatic dissociation using a DMEM solution containing 2.4U/ml dispase (Roche, Castle Hill), 2.4mM CaCl₂ and 1% w:v type II collagenase (Roche) at 2ml/g of tissue (Rando and Blau, 1994) incubated at 37°C for 45min. Myoblasts were proliferated at 37°C, 5% CO₂ in Ham's F-10 media (Invitrogen, Victoria) supplemented with 20% fetal bovine serum (Invitrogen), 0.5% chick embryo extract (US Biological, MA), 10U/ml penicillin (Invitrogen), 10µg/ml streptomycin (Invitrogen), 250ng/ml amphotericin B (Sigma, Castle Hill) and 500ng/ml basic fibroblast growth factor (Invitrogen). Human myoblasts were plated on 24-well
plates coated with 50µg/ml poly-D-lysine (Sigma) and 100µg/ml Matrigel (Becton Dickinson, New South Wales) and differentiated in DMEM supplemented with 5% horse serum (Invitrogen) for 3 days prior to transfection.

2'-O-methyl modified bases on a phosphorothioate backbone (2OMe AOs) targeting the human dystrophin transcript were synthesized in-house on an Expedite 8909 nucleic acid synthesizer (Applied Biosystems, Melbourne) using the 1µmol thioate synthesis protocol. 2OMe AOs were transfected in Opti-MEM using Lipofectamine 2000 (L2K) at a 1:1 (wt:wt) AO:lipoplex ratio according to manufacturer’s instructions (Invitrogen). The AO concentrations cited in the text and figures are for each individual AO within a cocktail.

4.2.2 PCR analysis of exon skipping

RNA was extracted from human myoblast cultures at times specified using Trizol (Invitrogen), according to the manufacturer’s instructions. A single step RT-PCR was performed on 100ng of total RNA for 35 cycles of amplification, using 1U of Superscript III (Invitrogen) in a 12.5µl reaction. The initial cDNA synthesis was performed for 30min at 55°C, before amplification with primers DMD1a and DMD1b described by Roberts et al (Roberts et al., 1992) at 94 °C for 30 sec, 55°C annealing for 1min, 72°C extension for 2min. A 1µl sample from this reaction was then used as the template for an additional 30 cycles of secondary amplification with DMD1c and DMD1d (Roberts et al., 1992) using 0.5U of AmpliTaq Gold (Applied Biosystems) under thermal cycling conditions described above. Products were then electrophoresed on a 2% TAE agarose gel, with products of interest purified using MoBio UltraClean spin columns (Geneworks, Adelaide) and then sequenced on an Applied Biosystems 377 automated sequencer using BigDye V3.1 terminator chemistry (Applied Biosystems).

4.3 Results and Discussion

4.3.1 Individual exon skipping

We, and others, have shown that approximately two thirds of AOs directed at the dystrophin gene transcript are capable of inducing some exon skipping (Aartsma-Rus et al., 2005, Wilton et al., 2007). However, some AOs are more efficient at removing targeted
exons than others and the combination of the most efficacious compounds would be assumed to lead to robust and consistent exon skipping. As several AOs would be required to remove multiple exons from the same dystrophin transcript, this necessitates that AOs must be effective at low concentrations. In turn, the desired biological action at low concentrations should reduce the possibility of non-antisense and off-target effects. To determine the optimal sequence, AOs were targeted to splice motifs and potential exon splicing enhancer (ESE) sequences, as predicted \textit{in silico} by ESE Finder (http://rulai.cshl.edu/tools/ESE2/) (Cartegni et al., 2003). ESE sequences are thought to serve as potential binding sites for serine/arginine-rich (SR) proteins (Blencowe, 2000). These proteins are a group of structurally related and highly conserved splicing factors that contain 1-2 RNA-recognition motifs (RRM) and a carboxy-terminal domain enriched in Arg/Ser dipeptides (RS domain) (Birney et al., 1993). SR proteins bind specifically to the RNA through the RRM domain and are proposed to promote exon recognition by either directly recruiting splicing machinery via the SR domain and/or repressing nearby silencer elements (Cartegni et al., 2002). Presumably, binding of AOs causes steric interference at these sites, disrupting the normal splicing function such that the exon fails to be recognized and is removed along with intronic sequence during pre-mRNA processing. ESE Finder2 predicts binding sites for four different SR proteins SF2/ASF, SC35, SRp40 and SRp55, calculating a value for sites throughout the pre-mRNA, based on homology to consensus motifs.

Motifs with high homology to SR binding scores were used as a basis for the initial AO design. For each of the exons targeted, a single AO was eventually developed that could induce exon skipping efficiently at concentrations of 25nM, 24 hrs after transfection \textit{in vitro} (Figure 4.1). Targeting of exon 8 induced the removal of exon 8 and 9 and has been reported previously (Aartsma-Rus et al., 2005, McClorey et al., 2006a, McClorey et al., 2006b, Wilton et al., 2007). This is not due to cross annealing of the AO but presumably reflects the close coordination of pre-mRNA processing. Targeting of exon 9 induces the removal of exon 9 only (Wilton et al., 2007), suggestive of some direction and order to the splicing process, with intron 9 excised prior to introns 7 or 8.

It should be noted that during the optimization process, several AOs induced poor or no detectable exon skipping. This was the case for exons 3 and 5, for which 9 and 10
different AOs respectively, were tested before an effective AO was produced. As in previous studies, targeting of predicted ESE sites had variable success, with no common motif identified that will reliably result in targeted exon skipping. The predicted values of putative SR sites expressed as ESE score minus the threshold value, for those regions targeted by the AOs used in this study, are listed in Table 4.1. Whilst the SF2/ASF protein motif is targeted by all of the AOs, targeting of this region alone was insufficient to induce exon skipping (data not shown). Likewise, none of the AOs targeted only a single ESE, but covered a region containing multiple predicted SR protein binding sites. This may suggest that more than one motif in an exon is involved in exon recognition, so that blocking one site may result in an alternative SR binding site being used. Given that it is not known which SR sites are most crucial for enhancing exon recognition, it may also be more advantageous to block multiple sites with the same AO. Higher ESE values for one particular SR protein did not indicate efficient AO design, with each SR protein having relatively high and low values spread throughout the different AO target regions. A previous study on AO design showed similar results whereby not every effective AO had high values for SR protein binding sites and some ineffective AOs did have high values (Aartsma-Rus et al., 2005). Another factor to consider in AO design is the presence of exonic splicing silencers (ESSs). Most silencers described are intronic and less well characterized, but several exonic ESSs have been reported (Amendt et al., 1995). These regions are thought to interact with negative regulators, such as the hnRNP I protein (also known as polypyrimidine-tract-binding protein) to mediate silencing of the exon (Wagner and Garcia-Blanco, 2001). Increasing the length of a very effective AO targeting mouse exon 23 donor splice site by 5bp upstream or downstream significantly reduced its ability to induce exon skipping, possibly due to obstruction of silencing elements (Harding et al., 2007). In contrast, when targeting exon 16 of the human dystrophin gene transcript, lengthening an AO of moderate efficacy by 6 nucleotides upstream or downstream increased its ability to induce exon skipping by more than an order of magnitude, presumably through targeting of additional ESEs (Harding et al., 2007). Thus, we have a system of positive and negative regulators, as well as the native consensus splice sites, determining whether an exon is included in the mature mRNA transcript. Therefore, once a region has been identified that could be targeted for exon skipping, fine tuning of the AO annealing position and/or length could increase the efficacy of exon exclusion.
4.3.2 *Multiple exon skipping*

Having designed AOs for the efficient removal of individual exons, we aimed to first induce the removal of exon subsets of 3-5 and 6-9, to validate the pattern of exon skipping for these smaller regions before attempting to induce excision of the larger exon 3-9 region. Inducing removal of smaller regions would also be applicable when addressing clustered protein truncating mutations that occur in this region. Targeting the dystrophin transcript with a cocktail of AOs for exons 3, 4 and 5 at a 1:1:1 ratio, induced a smaller dystrophin transcript, corresponding to the expected 883bp RTPCR product (Fig 4.2a). The shortened transcript was still present 7 days after transfection of the AO cocktail at 10nM concentration, indicating the efficiency with which these AOs induce multiple skipping *in vitro*. Similarly, targeting of exons 6-9 with AOs directed to exon 6, 7 and 8 at a 1:1:1 ratio was highly effective at inducing the expected 544bp RTPCR product (Fig 4.2b). The presence of intermediate products with variable exon removal was not unexpected and has been observed previously (Fall et al., 2006). Although any potentially unproductive out-of-frame transcripts would be subject to rapid nonsense mediated decay, they could reduce the overall level of dystrophin transcripts available for correction. If these intermediate products were the dominant transcript, further optimization of the AO design would be necessary. However, 7 days following transfection, the in-frame Δ8-9 and Δ6-9 are the predominant skipped transcripts and so further optimisation was not deemed necessary.

Having induced consistent skipping of the exon 3-5 and 6-9 subsets, the dystrophin pre-mRNA was targeted with AOs to remove exons 3-9. A 274bp transcript representing exon 2 spliced to 10 was detected in cultures treated for 1-7 days, at all AO concentrations tested, and in the untreated samples (Figure 4.3). This transcript was observed in untreated controls from multiple primary human cell lines and is likely to be an endogenous alternatively spliced transcript. It has been described previously in BMD patients with exon 3-7 deletions and in normal muscle (Wilton SD, unpublished observations) (Chelly et al., 1991, Chelly et al., 1990). Variable abundance of this particular gene transcript may explain why some patients with the 3-7 or 5-7 exon deletions sometimes present with a less severe phenotype than would have been expected for a protein truncating mutation. Dystrophin protein translated from these alternative transcripts may be produced in sufficient amounts to partly ameliorate the condition. As this transcript is indistinguishable from that induced by exon skipping, interpretation of the efficacy of this approach was
difficult. It could be speculated that any of the A3-9 transcript present in the treated samples is endogenous rather than a product of AO action. However, we propose that it is more likely a combination of both endogenous alternatively spliced transcripts and AO induced shortened transcript for the following reasons. There was a visible reduction in the amount of full-length dystrophin transcript in treated cells compared to untreated cells in a dose-dependent manner. Additionally, multiple transcripts intermediate between the A6-9 transcript and the A3-9 transcript, which are not present in the untreated samples, were detected in the treated samples. These transcripts are missing almost all the target exons, suggesting that AOs are removing more than just the A6-9 subset and are probably indicative of a spectrum of transcripts whereby five or six exons are being excluded. The 212bp transcript observed in the untreated sample at day 1 represents a A2-9 dystrophin transcript that was not detected in cells transfected with AO (Fig 4.3a). Over time, this transcript became more predominant, until by days 5 and 7, it is present in almost all samples. This would suggest that the formation of the A3-9 transcript is biased by AO action over the naturally occurring A2-9 transcript that has also been identified previously (Chelly et al., 1990). As the 20MeAOs are metabolized and gradually degraded over time in culture, the increase in the ratio of A2-9 to A3-9 dystrophin transcripts may be further evidence of a genuine AO-mediated effect. A dose dependant effect is evident in days 3 to 7 of culture, with consistently stronger induction of the A3-9 transcript following AO treatment in comparison with untreated cultures. However, these observed transcript levels are not significantly higher than endogenous A3-9 levels, and this may reflect a bias in the PCR detection assay due to preferential amplification of shorter products. If the AO treatment could be optimized to reduce the amount of intermediate transcripts, by either varying the AO cocktail ratios or by increasing the efficacy of the AOs targeting the A3-5 transcript, the level of A3-9 dystrophin transcript may increase sufficiently for this approach to be beneficial. The toxic effects of 20Me AO lipoplexes limited the use of higher concentrations of AO cocktail to determine if increased dosage would significantly improve A3-9 transcript levels. An alternative approach would be to use a different chemistry, such as PMOs, that exhibit fewer adverse effects and should allow higher AO concentrations to be applied.

Exon skipping has emerged as a promising approach to address DMD causing mutations. In particular, the ability to design of AO ‘cocktails’ to skip multiple exons has
the potential to develop a single treatment that would address several different DMD mutations, simplifying the progression to clinical treatment. Inducing dystrophin exonic combinations that correspond to known mild phenotypes would be preferable, given that the induced protein would be expected to be functional, allowing treatment of clustered mutations. Previous studies exploring multiple exon skipping focused on targeting the outermost exons of the region to be skipped, with limited success (Aartsma-Rus et al., 2006). Targeting the individual removal of exons 3-9, which corresponds to a very mild BMD phenotype, showed that this transcript could be induced using this approach. However, whilst multiple exon skipping is possible, efficacy was low and further optimization of AO cocktail design or the use of alternative AO chemistries may be necessary in order to provide a measurable clinical benefit. Although in those situations where one or more exons are already missing, such as for genomic deletions, a cocktail of AOs to induce multiple exon skipping could be expected to be more beneficial. There is also the consideration that there may be a limit to the number of exons that can be targeted before inducing adverse effects on the cellular splicing process. Using AOs to artificially remove such a large fragment of pre-mRNA could disrupt the splicing process in this region, to the extent that re-initiation of normal splicing would be affected. This should be an important consideration when determining the optimal exon skipping approach for each individual. Until the process of multiple exon skipping is better understood, continuing optimization of treatments to address the minimal changes required to overcome DMD mutations should remain the focus for clinical trials.

4.4 References


<table>
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<th>Nomenclature</th>
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Table 4.1 – Sequences and relative position of antisense oligonucleotides used in this study and the predicted values of SR protein ESE motifs targeted by these oligonucleotides. Bracketed numbers refer to position of oligonucleotide relative to acceptor splice site with “+” referring to position within exon and “-” referring to position within intron. ESE Finder values displayed are corrected for SR protein variation by subtraction of minimum threshold values.
Figure 4.1 Titration of high efficacy 2OMeAOs designed to target removal of individual exons from the dystrophin transcript. (a, b, c) Amplification across exon 1-6 (669bp) showing exon 3 (576 bp), exon 4 (591 bp) and exon 5 (576 bp) AO induced skipping respectively. (d, e, f) Removal of exon 6 (1013 bp), exon 7 (1067 bp) and exon 8/9 (869 bp) from an exon 1-10 PCR product (1186 bp). Δ indicates excluded exons. UT indicates untreated.
Figure 4.2 Nested PCR amplification from exon 1-10 (1186 bp) showing time course of multiple exon skipping of subsets of (a) exons 3-5 (922 bp) and (b) exons 6-9 (577 bp) from the dystrophin transcript at 1, 3, 5 and 7 days. AO concentration shown is for each individual AO, with uncomplexed treatment (100nM naked) referring to transfection in the absence of cationic liposome. Δ indicates excluded exons. UT indicates untreated.
Figure 4.3 Nested PCR analysis of induction of the Δ3-9 transcript from the dystrophin mRNA showing Δ6-9 (577 bp) and Δ3-9 (313 bp) as predominant transcripts. Δ indicates excluded exons. UT indicates untreated.
5.1 Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disease that is typically caused by nonsense or frame-shifting mutations in the dystrophin gene that result in the loss of functional protein (Hoffman et al., 1987). Antisense oligonucleotides (AOs) have been used to modulate dystrophin pre-mRNA splicing to reduce the consequences of nonsense or frame-shifting mutations that would otherwise have prematurely terminated translation. AOs can redirect splicing patterns by masking motifs in the dystrophin pre-mRNA to block spliceosome assembly, such that the target exon is not recognized by the splicing machinery (Aartsma-Rus et al., 2003, Mann et al., 2001). In this manner, one or more targeted exons can be removed, with the flanking intronic regions, from the mature RNA. Selected removal of a nonsense mutation or restoration of the reading-frame around a genomic deletion or duplication should result in an mRNA transcript that can be translated into an internally shortened, yet still partially functional dystrophin. In many cases, it should be possible to predict the level of functionality of the induced dystrophin protein by comparing it with analogous rearrangements found in Becker muscular dystrophy.
dystrophy, a less severe condition generally arising from in-frame dystrophin deletions (Comi et al., 1994).

The use of AO-induced dystrophin exon skipping has rapidly progressed from \textit{in vitro} mouse (Mann et al., 2001) and human (van Deutekom et al., 2001) studies using cultured cells, to \textit{in vivo} application in animal models (Lu et al., 2003, Lu et al., 2005) and finally to preparation for human clinical trials (Muntoni et al., 2005). Parallel human trials are currently envisaged where two different AO chemistries, 2’-O-methyl modified ribose moieties on a phosphorothioate backbone (2OMe AO) and phosphorodiamidate morpholino oligonucleotides (PMO), will be directed at amenable domains in the dystrophin pre-mRNA to induce exon skipping.

Phase I trials will involve intramuscular administration of limited amounts of the compounds into dystrophic muscle (Muntoni et al., 2005). It is generally acknowledged that intramuscular delivery regimens are not a viable clinical option for AO induced exon skipping, since dystrophin exon skipping will only be localized and persist for the duration of the biological activity of the oligonucleotides. A comprehensive series of intramuscular injections across the body to address all dystrophic muscles, including the heart, will not be applicable in a clinical setting, particularly since periodic readministration would be needed. As 2OMe AOs have not previously been injected into humans, initial safety and toxicology studies will be mandatory. It is proposed that the initial 2OMe AO studies in DMD patients will commence with administration by intramuscular injection (Muntoni et al., 2005). Although relatively small amounts of 2OMe AO will be injected into dystrophic muscle, compared to that necessary for systemic treatment, localized AO concentrations will be far higher than those which could be achieved after systemic delivery. Whilst successful induction of localized dystrophin expression would demonstrate proof of concept in human muscle, only limited information could be obtained from around the injection site with respect to safety, AO distribution and duration of induced dystrophin expression.

In contrast to the 2OMe AO chemistry, PMOs have already undergone several human clinical trials where PMOs with different targeted sequences have been systemically administered to humans in treating restenosis, cancer (Devi et al., 2005) and viral
infections, with no drug-related adverse effects observed to date (http://www.antivirals.com/devNeugene.html). However, for PMOs conjugated to peptides for enhanced cellular delivery (PMO-Peps), initial safety trials will be necessary as they have yet to be used in humans, although testing is ongoing in rodent and primate models. We have recently shown in vivo that PMOs induce more sustained exon skipping in the mdx mouse model of muscular dystrophy than the equivalent 2OMe AO (Fletcher et al., 2005). Proof that any AO, of either the 2OMe AO or PMO chemistries, can induce dystrophin exon skipping in vivo in humans has not ye; been demonstrated.

In an attempt to minimize the number of intramuscular studies needed to provide proof of concept, we describe an ex vivo system to evaluate induced exon skipping in muscle. Hasson et al 2005, described the potential of micro-organs; microscopic fragments that preserve the tissue structure of their organ of origin, as a method for monitoring viral transfection and gene replacement (Hasson et al., 2005). We show here that muscle fragments, obtained after informed consent during unrelated, elective surgical procedures, can be transfected with AOs to induce specific exon skipping for up to two weeks. To our knowledge, this is the first time that AO induced exon skipping in human muscle has been reported.

5.2 Materials and Methods

5.2.1 Sources of tissue

Mouse muscle was taken from the tibialis anterior of 6 week old mdx mice. Human muscle biopsies were obtained from normal individuals undergoing elective surgery at Royal Perth Hospital after informed consent. Dystrophic human muscle was obtained from a DMD patient undergoing spinal corrective surgery, following informed consent at Childrens Hospital Westmead, Sydney. The dystrophin mutation in this patient had been identified as an exon 3-17 deletion, which was subsequently confirmed in the exon skipping studies. Human muscle was stored in DMEM (Invitrogen, Melbourne) with 20% FBS (Invitrogen) and 50U/ml penicillin (Invitrogen), 50μg/ml streptomycin (Invitrogen) and 1.25 μg/ml amphotericin B (Sigma, Castle Hill, Australia) for approximately 2 hours at 4°C prior to use, whilst the DMD muscle was shipped on ice overnight in the same media.
5.2.2 Antisense oligonucleotides

The 2OMe AO, M23D(+07-18), was obtained from Avecia (Grangemouth, Scotland). AOs targeting human dystrophin gene transcripts were synthesized in-house on an Expedite 8909 nucleic acid synthesizer (Applied Biosystems, Melbourne) using the 1µmol thioate synthesis protocol. PMO were synthesized at AVI BioPharma, USA as described elsewhere (Summerton and Weller, 1997). The cell penetrating peptide conjugated to PMO has the sequence of (RXR)4XB where X and B represent 6-aminohexanic acid and beta-alanine, respectively. The peptide was synthesized using standard FMOC chemistry and purified to >90% purity at AVI BioPharma.

5.2.3 Exposure of tissue explants to antisense oligonucleotides

Muscle was dissected into small fragments, approximately 2-3 mm³ and infused in an OptiMEM (Invitrogen, Australia) -AO solution with 50U/ml penicillin, 50µg/ml streptomycin and 1.25 µg/ml amphotericin B, containing either uncomplexed 2OMe AOs, PMOs or PMO-Pep. AOs were directed to either dystrophin exon 23 for mouse studies, exons 6 or 8 for normal human muscle studies or exon 18, 19 and 20 for DMD muscle studies (Table 5.1). The nomenclature for AOs is described by Mann et al (Mann et al., 2002). Muscle fragments were incubated in the OptiMEM-AO solution at 37°C, 5% CO₂, and after 3 days were supplemented with an equal volume of DMEM containing 10% horse serum, to obtain a final concentration of 5% horse serum.

5.2.4 PCR analysis of exon skipping

RNA was extracted from free-floating muscle explant fragments at specified times using Trizol (Invitrogen), according to the manufacturer’s instructions. RT-PCR was performed on 100ng of total RNA for 35 cycles of amplification, using 1U of Superscript III (Invitrogen) in a 12.5µl reaction. Primers were used at 94 °C for 30 sec, 55°C annealing for 1min, 72°C extension for 2min and are listed in Table 5.2. A 1µl sample from this reaction was then used as the template for 30 cycles of secondary PCR amplification using 0.5U of AmpliTaq Gold (Applied Biosystems) under cycling conditions described above. Products were then electrophoresed on a 2% TAE agarose gel, with products of interest purified using MoBio UltraClean spin columns (Geneworks, Adelaide) and then sequenced.

5.3 Results

5.3.1 Optimisation of procedure

Muscle from mdx mouse tibialis anterior was used to optimize the technique for AO induced exon skipping in muscle explants which were either injected, infused, or injected and infused with 5μM of M23D(+07-18) 2OMe AO, PMO or PMO-Pep in OptiMEM (data not shown). RNA was then extracted 1 or 7 days later for nested PCR analysis. Removal of exon 23 (Δ213bp) or 22 and 23 (Δ359bp) from the dystrophin transcript could be detected after 24h (data not shown), however exon skipping was more sustained and pronounced following 7 days exposure to the AO (Fig 5.1). Infusion in the AO-OptiMEM solution was preferable, as this technique was more easily controlled, unlike injections, where leakage of the AO solution could occur from some small muscle explants. As no advantage was conferred by combining AO injection with infusion, AO infusion alone was selected as the optimum condition to induce exon skipping in muscle explants.

5.3.2 Single exon skipping in explants

AO induced exon skipping was demonstrated in normal human muscle explants, comparing the efficacy of the three AO chemistries. A biopsy fragment from the vastus medialis muscle was dissected into 2-3 mm³ segments and infused with 10μM of either H6A(+69+91) or H8A(-06+18) 2OMe AO, PMO or PMO-Pep for 7 days prior to RNA extraction. H6A(+69+91) or H8A(-06+18) AOs had been optimized in our laboratory and were the only human dystrophin AOs available that had been synthesized using all three chemistries. Removal of exon 6 (Δ173bp) from the dystrophin transcript was detected for each AO chemistry with an observed efficacy: PMO-Pep > PMO > 2OMe in the ability to induce exon removal (Fig 5.2). Removal of exons 6 and 9 (Δ305bp) from the dystrophin transcript was sporadically observed (Fig 5.2). In contrast, exon 8 skipping (Δ185bp) was always accompanied by exon 9 removal and could be detected in PMO and PMO-Pep treated tissue, but not after treatment of the explant with the 2OMe AO (Fig 5.2).
Dystrophic muscle was obtained from a DMD patient with the deletion of dystrophin exons 3-17, which produces an out-of-frame transcript that precludes the synthesis of a full length dystrophin protein. Removal of exon 18 restores the dystrophin mRNA reading frame and would potentially allow the synthesis of an internally deleted dystrophin protein. H18A(+24+53) and H18A(+31+61) had been designed to induce exon 18 removal based on *in vitro* exon skipping studies using normal human myoblast cultures (data not shown). These AOs were only available as 20Me compounds, so PMO and PMO-Pep chemistries could not be evaluated. DMD muscle explants were infused with H18A(+24+53) or H18A(+31+61) 20Me AO at 10µM, 20µM and 40µM and RNA extracted for nested PCR analysis after incubation for up to 14 days. Both AOs induced a similar pattern of exon 18 removal, but only the results for the H18A(+24+53) AO are shown (Figure 5.3A-C). Strong and consistent levels of exon 18 skipping was observed by day 3 (Figure 5.3A) induced at all concentrations, and was still evident at day 14 (Fig 5.3C). Sequencing of the shorter PCR product confirmed precise splicing of exon 2 to exon 19 (Fig 5.3D).

5.3.3 *Multiple exon skipping in explants*

To ascertain if multiple exon skipping could be induced, a cocktail of three additional AOs designed to remove exons 19 and 20 were tested. A 10µM cocktail of H19A(+35+65), H20A(+44+71) and H20A(+147+168) 20Me AOs was combined with 10µM of H18A(+24+53) and DMD tissue fragments incubated with the AO mix for 1, 3, 7 and 14 days. No exon skipping was observed following 1 or 3 days of infusion, however by days 7 and 14, removal of exon 20 alone (Δ242bp), 18 and 19 (Δ212bp) and 18, 19 and 20 (Δ454bp) was observed (Fig 5.4A). The appearance of the shorter transcript correlated with a reduction in the amount of full length transcript, with the in-frame transcript arising from splicing of exon 2-21 being the dominant transcript at day 14 (Fig 5.4A), as confirmed by sequence analysis (Fig 5.4B).

5.4 *Discussion*

Recent studies in the *mdx* mouse and in human muscle cultures have clearly established the potential of an AO induced exon skipping approach to restore dystrophin synthesis despite mutations in the dystrophin gene. To date however, these studies have
been limited to animal models and *in vitro* human experiments, which although promising, are unable to provide conclusive evidence that exon skipping would be induced when AOs were delivered to human muscle.

A humanized mouse model has been constructed to facilitate direct testing of AOs to induce exon skipping within the human dystrophin gene transcript in muscle (Bremmer-Bout et al., 2004). However, we urge some caution in interpreting data obtained during the processing of a human gene transcript using rodent splicing machinery. Whilst we have shown that the same AOs can induce exon 19 skipping in human and murine cells *in vitro* (Errington et al., 2003), we have found that targeting the coordinates which resulted in strong and sustained exon 23 skipping in the *mdx* mouse, had no effect on human exon 23 retention *in vitro* (unpublished observations). Similarly, when the same coordinates that excluded human dystrophin exons 52 and 53, were targeted in the *mdx* mouse, there was no effect on murine dystrophin mRNA processing (unpublished observations).

A splice mutation in the sodium channel Naᵥ1.6 gene (*Scn8a*) particularly highlights the importance of splicing machinery. Two strains of mice with different splicing backgrounds process the mutant transcript differently and show dramatic variation in disease phenotype (Nissim-Rafinia and Kerem, 2005, Buchner et al., 2003). Additionally, phenotypic variation can arise within families with the same dystrophin mutation. Disease severity in a family with a nonsense mutation in exon 29, appeared to correlate with the levels of exon 29 skipping (Ginjaar et al., 2000). In the presence of such reports, we would suggest that it is generally preferable to monitor human dystrophin splicing on a human background.

Currently proposed clinical trials will initially undertake localized intramuscular injections to provide proof of concept in human muscle (Muntoni et al., 2005). Obviously, systemic AO delivery would be preferable, but in the absence of proof-of-principle of this approach in human tissue, approval for systemic clinical trials is unlikely to be forthcoming, particularly for those AO chemistries that have not yet been used in humans. To address this issue, we sought to develop an *ex vivo* system that would allow for the testing of AO analogues to induce exon skipping in human muscle.
Due to the precious nature and limited availability of human tissue, optimization of this approach was initially performed using \textit{mdx} mouse muscle. Accurate injection of AOs into the muscle fragments was hampered by AO leakage from the explant, reflected in variable induction of exon skipping (data not shown). In contrast, tissue infusion in media containing the AO at a specified concentration allowed for more consistent AO delivery in multiple explants with typical results presented. Additionally, AO incubation allowed the use of smaller muscle fragments, which maximized the surface area of muscle fibres in contact with the AO.

Previous \textit{in vitro} (Mann et al., 2001) and \textit{in vivo} (Lu et al., 2003, Lu et al., 2005) studies demonstrated that targeting the donor site of exon 23 could efficiently induce removal of that exon and by-pass the \textit{mdx} nonsense mutation. Incubation of \textit{mdx} tissue in the 20Me, PMO and PMO-Pep AOs preparations induced the removal of exon 23 and exons 22 and 23 from the dystrophin transcript. The removal of exon 22 and 23 has been reported previously (Mann et al., 2001) and is thought to reflect closely coordinated processing of these exons. The level of exon skipping was higher and more consistent following 7 days of infusion, indicating that explant viability was sufficient to allow the splicing process and subsequent synthesis of dystrophin mRNA to continue (data not shown). All AO chemistries induced strong exon skipping where at least 50% of the transcripts were missing exon 23, and under these conditions it is not possible to identify the preferred compound. Differences in exon skipping efficacy would become more apparent at lower AO concentrations and over extended time points. We have recently shown that PMOs were able to exert more persistent and sustained exon 23 skipping in the \textit{mdx} mouse \textit{in vivo} when compared to the equivalent 20MeAO (Fletcher et al., 2006).

Having induced consistent exon removal in \textit{mdx} tissue, the approach was extended to normal human muscle. Surplus muscle from individuals undergoing contracture testing for malignant hyperthermia was obtained for explant studies. Similarly, we would only obtain dystrophic material when a patient undergoes elective surgery. These restrictions limit the availability of muscle for experimentation; however no patient would be required to undergo surgery only for these studies. We do not consider this \textit{ex vivo} protocol to be practical for pre-screening patients for suitability for an exon skipping strategy.
We have developed a panel of AOs to address mutations across the human dystrophin gene transcript (Wilton et al., 2005). Because of our interest in the minor deletion hotspot of the human dystrophin gene (Baumbach et al., 1989), and also in addressing the golden retriever muscular dystrophy (GRMD) mutation (McClorey et al., 2006), we had AOs optimized for exons 6 and 8 that had been synthesized as all three AO preparations, 20Me, PMO and PMO-pep. All three AO compounds induced exon 6 removal whereas only PMO and PMO-pep induced exon 8 (and 9) removal consistently at 10μM. The 20Me AO was unable to excise exon 8 (and 9) for multiple explants treated at 5, 10 or 20 μM despite being efficiently induced in vitro (Wilton et al., 2005). The inability of this compound to induce exon skipping in explants may indicate the need for a higher dosage in order to induce a comparable response to that shown with the PMO and PMO-Pep. Alternately, the environment of the explant tissue may more closely resemble the in vivo model and as such may behave differently to typical in vitro results. Fletcher et al (Fletcher et al., 2006), demonstrated in the mdx mouse that a 20Me AO directed at the exon 23 donor splice site was very inefficient at inducing exon skipping in vivo, when compared to the PMO under the same delivery conditions.

Significantly, the removal of either exon 6 or exon 8 from the normal dystrophin transcript produces an out-of-frame transcript, which would be expected to be subjected to nonsense mediated decay (Maquat, 2005). It is testament to the efficacy of this approach that substantial levels of these transcripts could be observed relative to the normal full length human dystrophin transcript. The removal of exon 8 was always accompanied by the removal of exon 9 from the transcript, a feature also observed in GRMD (McClorey et al., 2006) and in vitro human studies (Aartsma-Rus et al., 2005). Non-specific exon 9 removal has also been observed in mouse and human tissue (Reiss and Rininsland, 1994). This alternative splicing is thought to be due to the 3’end of exon 9 resembling an intronic 3’ splice site, such that it is “identified” as an intron and removed from the mRNA transcript (Reiss and Rininsland, 1994). This could account for the sporadic removal of exon 9 that was observed when exon 6 was targeted. Removal of exon 9 does not change the mRNA reading frame and is not expected to be detrimental. These experiments demonstrate a proof of principle that AOs can be used to induce targeted exon skipping in dystrophin transcripts in human muscle.
The pathology of DMD muscle is substantially different from normal muscle (Emery, 1993) and to provide further validation of this approach, AO induced exon skipping was again demonstrated in human DMD muscle. An exon 3-17 deletion produces an out-of-frame transcript that could be corrected by removal of exon 18. To address this mutation, the H18A(+24+53) or H18A(+31+61) 2OMe AOs facilitated exon 18 exclusion to induce an in-frame transcript, the level of which was highest after 14 days. Unfortunately, only 2OMe AOs were available to target exon 18, so a comparison of AO chemistry efficacy could not be made. The presence of high levels of in-frame transcripts indicates that the human muscle fragment is retaining the ability to transcribe dystrophin mRNA after 2 weeks. Removal of additional exons 19 and 20 was undertaken simply to demonstrate the robust nature of exon skipping, and show that multiple exon skipping could be induced in muscle explants, as has been previously demonstrated in human DMD myotube cultures (Aartsma-Rus et al., 2004). Whilst removal of a single exon could exclude the mutation or restore the reading frame for the majority of DMD gene lesions, certain mutations will require removal of multiple exons. For example, a nonsense mutation in exons 6, 7, 8 or 9 would necessitate the removal of all those exons to by-pass the premature termination signal and restore the reading frame. In some instances it may actually be preferable to remove multiple exons if the resultant dystrophin transcript corresponded to that of one of the Becker muscular dystrophy patients, who have larger in-frame deletions associated with a mild phenotype (Love et al., 1990). A cocktail of three AOs, in combination with H18A(+24+53) was able to induce the removal of exons 18, 19 and 20 to produce a smaller in-frame transcript. Two AOs were required to induce the most efficient removal of exon 20 (Wilton et al., 2005). The level of this induced transcript was again highest following 14 days infusion, presumably reflecting the accumulation of an in-frame transcript that would not be subject to nonsense-mediated decay. It was unexpected that multiple exon skipping was not observed until day 7, given that skipping of exon 18 alone was shown after 3 days. However, it should be noted that the exon 18 skipping tended to become more pronounced with time and the differences in the relative amounts of exon skipping may reflect variability in the starting material.

There was insufficient material to attempt Western blotting analysis and the condition of the explant was such that immunostaining was not practical. Studies on subcutaneous implanted mouse explants indicated initial degeneration before active
regeneration resulting in the formation of a new population of muscle fibres (Mastaglia et al., 1975). In response to the mechanical damage and culturing conditions, we would expect necrosis and activation of myogenic cells to occur within the muscle explants, not dissimilar to dystrophic tissue. It could be predicted that the in-frame dystrophin transcripts arise from a combination of fibres already present in the tissue and fibres regenerating from a population of mononuclear cells. We acknowledge limitations of the ex vivo manipulation but suggest this could provide an intermediate model between cultured cells and human in vivo studies.

Although this study demonstrated that 2OMe AOs could induce exon skipping in normal and DMD human muscle, these compounds have yet to be injected into humans and so initial studies using this chemistry would necessitate localized intramuscular injections. The PMO chemistry on the other hand, has been administered systemically to over 250 patients with no reported adverse effects. As PMOs have been shown here to induce exon skipping in human muscle, we provide the proof of concept to cautiously suggest that it may be possible to move directly to human systemic clinical trials with this chemistry, or at least minimize the number of participants in intramuscular studies. A systemic study with repeated administration would not only provide more useful information about the applicability of an AO based approach to treat DMD but more importantly, may be therapeutically more beneficial to the participants in these trials.

5.5 References


<table>
<thead>
<tr>
<th>Description</th>
<th>Nomenclature</th>
<th>Sequence 5'–3'</th>
</tr>
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<tbody>
<tr>
<td>Mouse Exon 23 2OMe AO</td>
<td>M23D(+07-18)</td>
<td>caaaccucgcuuacCUAGAAAU</td>
</tr>
<tr>
<td>Human Exon 6 AO</td>
<td>H06A(+69+91)</td>
<td>UACGAGUGUAUGUCGGACCCAG</td>
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<tr>
<td>Human Exon 8 AO</td>
<td>H08A(-06+18)</td>
<td>GAUAGGUGUAUCAACAUcuguaa</td>
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<td>Human Exon 18 AO</td>
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<td>CAGCUUCUGAGCGAGUAUCCAGCUGUGAA</td>
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<tr>
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<tr>
<td>Human Exon 20A AO</td>
<td>H20A(+44+71)</td>
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<tr>
<td>Human Exon 20B AO</td>
<td>H20A(147+168)</td>
<td>CAGCAGUAGUUGUCAUCUGCUC</td>
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**Table 5.1** – Sequences of antisense oligonucleotides used in this study. Upper and lower case characters indicate exonic and intronic nucleotides respectively. PMO chemistries have uracil bases substituted with thymine.

<table>
<thead>
<tr>
<th>Description</th>
<th>PCR primer</th>
<th>Sequence 5'–3'</th>
<th>Expected size (bp)</th>
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<td>Mouse Exon 13-27 Outer</td>
<td>MExon13F</td>
<td>gcttcaagaagatctagaacaggagc</td>
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<td></td>
<td>MExon27R</td>
<td>cttattcagctctcagtaagg</td>
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<tr>
<td>Mouse Exon 18-26 Inner</td>
<td>MExon18F</td>
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<td>1286</td>
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<tr>
<td></td>
<td>MExon26R</td>
<td>tctttcagctgtgtgctatcc</td>
<td></td>
</tr>
<tr>
<td>Human Exon 1-10 Outer</td>
<td>HExon01F(a)</td>
<td>ctttccccctacaggactcg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HExon10R(a)</td>
<td>cttctcatcataagactgctc</td>
<td></td>
</tr>
<tr>
<td>Human Exon 1-10 Inner</td>
<td>HExon01F(b)</td>
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<td>1147</td>
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<td></td>
<td>HExon10R(b)</td>
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<tr>
<td>Human Exon 1-27 Outer</td>
<td>HExon01F(a)</td>
<td>ctttccccctacaggactcg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HExon27R</td>
<td>gctatgacactattacgactcg</td>
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<tr>
<td>Human Exon 1-26 Inner</td>
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<tr>
<td></td>
<td>HExon26 R</td>
<td>attcgtgcattctcgcgtgta</td>
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</tr>
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</table>

**Table 5.2** – Primer sequences for nested PCR analysis. * DMD exon 3-17 deletion taken into account when calculating expected size.
Figure 5.1 RT-PCR analysis of *mdx* mouse muscle explants following 7 days incubation with 10μM of either 2OMe, PMO or PMO-Pep M23D(+7-18) AOs. The shorter PCR products of 1073bp or 927bp, indicates removal of exon 23 or exon 22 and 23 respectively, from the dystrophin transcript. (Δ = exon removal).
Figure 5.2 Control human muscle explants were incubated with 10μM of either H6A(+69+91) or H8A(-06+18) 2OMe, PMO or PMO-Pep AOs for 7 days. Shorter PCR products indicate removal of exon 6 alone (974bp), exon 6 and 9 (842bp), exon 8 and 9 (830bp) or exon 9 alone (1015bp) from the dystrophin transcript.
Figure 5.3 Induction of exon 18 skipping in dystrophin transcripts from DMD muscle explants missing exon 3-17, to restore the mRNA reading frame. Explants were incubated with H18A(+24+53) 2OMe AO for (a) 3 days, (b) 7 days and (c) 14 days at 10, 20 and 40μM. A shorter PCR product of 1487bp corresponds to removal of exon 18 from the dystrophin transcript. A sporadic PCR product of ~1350bp could not be identified and is possibly a PCR artifact. PCR products larger than 1611bp are products of the outer primer set from the nested amplification. (d) Confirmation of the precise splicing of exon 2 to 19 was confirmed by sequencing.
Figure 5.4 (a) Multiple exon skipping in DMD Δ3-17 muscle explants incubated with 10μM of H18A(+24+53), H19A(+35+65), H20A(+44+71) and H20A(+147+168) 2OMe AOIs. RTPCR analysis was performed following 1, 3, 7 and 14 days incubation. Removal of exon 18 and 19 (1399bp), exon 20 (1369bp) and exon 18 and 19 and 20 (1157bp) could be observed by day 7 and 14. (b) Sequencing confirmation of precise removal of exons 3-20 from the dystrophin transcript.
Chapter 6

6.1 Discussion

As early as 1978, antisense oligonucleotides (AOs) have been used as a tool to down-regulate gene expression by annealing of AO to target mRNA (Zamecnik and Stephenson, 1978), which was later determined to be due to RNase-H mediated cleavage (Walder and Walder, 1988). Alternatively, gene down-regulation can also be achieved by steric blockage of translation initiation or elongation through modification of the ribose moiety such that RNase H activity is not activated (Chiang et al., 1991). Pioneering work by Richard Kole (Chapel Hill, North Carolina) demonstrated that these modified AOs could also be applied to address splicing mutations in the β-globin gene that were responsible for sickle cell anaemia through masking of cryptic splice sites such that splicing patterns were normalised (Dominski and Kole, 1993). This concept was soon applied to Duchenne muscular dystrophy (DMD) where it was known that internally deleted dystrophin protein was still functional if crucial terminal domains were retained. Thus it was proposed that manipulation of normal dystrophin splicing through targeting of exonic splice sites could be used to remove or compensate for the disease-causing mutations. This principle of AO induced exon skipping was first demonstrated in 1996 through targeted removal of exon 19 from lymphoblastoid cells (Pramono et al., 1996) and was subsequently applied in the mdx mouse model (Dunckley et al., 1998, Wilton et al., 1999) and in human DMD patient cells in vitro (van Deutekom et al., 2001) to by-pass protein truncating mutations. These initial studies paved the way for a rapid expansion of knowledge in this field, of which the work in this thesis has made a significant contribution. As stated in the introduction, this thesis proposes two main hypotheses. Firstly, that an AO mediated splicing modification approach can be adopted to address a clinically relevant animal model of DMD. Secondly, that further studies into exon skipping in a human model will lead to a greater understanding of the challenges facing this approach as well as contributing to the work of knowledge necessary to facilitate movement towards human clinical trials.

The first hypothesis was that an AO based exon skipping approach could be applied to a clinically relevant animal model. The evidence for this is presented in Chapters 2 and
3 where AOs were used to restore dystrophin expression in the Golden retriever muscular dystrophy (GRMD) model. The majority of research in the DMD field to date has utilised cultured human cells or the mdx mouse model, which exhibits a mild clinical phenotype relative to the human condition. Whilst these models are useful for demonstrating dystrophin restoration at a molecular and cellular level, it may be preferable to validate any potential therapeutic approach in an animal model which more closely resembles the DMD phenotype. The GRMD canine model, with its clinically similar pathological course and closer proximity in size to young DMD patients, is an excellent model for this, although it should be noted that it is not without its own limitations, such as high maintenance cost and relative scarcity. As well as demonstrating the proof of principle of this approach in the GRMD model, the work presented in Chapter 2 had significance for the rest of the studies in this thesis.

The first significant finding was that unlike the mdx mouse model, where targeting of the donor splice site was sufficient to induce exon removal, targeting of this splice site in either exon 6 or 8, could not induce their excision from the transcript. Whilst targeting of the acceptor splice of exon 8 induced its removal, it was necessary to block a putative exon splicing enhancer (ESE) sequence within exon 6 to facilitate efficient removal of that target. This finding was important, as it changed the approach made to inducing exon skipping in further dystrophin exon skipping targets. Previous AO design in our laboratory had predominantly focused on targeting of consensus splice sites, but these results suggested that ESE’s may be equally important, if not more so, in determining recognition of exons by splicing machinery. This was alluded to previously in the first demonstration of dystrophin exon skipping whereby AOs were targeted to a 31bp ESE region in exon 19 (Pramono et al., 1996), and also in human in vitro studies where a panel of overlapping AOs were directed across an exon to target potential ESE sites (van Deutekom et al., 2001). In early human in vitro studies, AOs were directed against purine-rich regions of predicted open secondary structure (Aartsma-Rus et al., 2002). However, for removal of exon 6, and later targeting of exons across the entire human dystrophin gene transcript (Wilton et al., 2007), in silico prediction with a web-based program, ESEFinder 2
(http://rulai.cshl.edu/tools/ESE2/) (Cartegni et al., 2003), was used to identify binding sites for splicing proteins involved in exon recognition.

For induced exon skipping to be clinically relevant, it is crucial that AOs are designed to be efficacious at the lowest possible concentrations. This is especially important when considering that for the GRMD model, and indeed for many human DMD mutations, more than one exon may need to be excluded. In these studies, restoration of the GRMD reading frame could be achieved at concentrations as low as 25nM \textit{in vitro}. This compares favourably in the context of previous studies, whereby AO concentrations of 200nM were used to induce multiple exon skipping in human patient cells (Aartsma-Rus et al., 2004). By optimizing the efficiency of these AOs \textit{in vitro}, it maximizes their potential for success \textit{in vivo}, where it is likely that only low levels of AO would be distributed to skeletal muscle tissue, especially when adopting a systemic approach.

One of the more novel and significant aspects of the GRMD studies was the comparison of efficacy of various AO chemistries to correct the GRMD model both \textit{in vitro} and \textit{in vivo}. At the commencement of this PhD study, the predominant chemistry available for study was 2’-\textit{O}-methyl ribose modified bases on a phosphorothioate backbone (2OMe). Both of these AO modifications have been shown to confer improved stability and resistance to nuclease degradation and its ability to induce exon skipping had been well established in both mouse and human cell models (Lu et al., 2003, Mann et al., 2001, van Deutekom et al., 2001). Importantly, these AOs could also be synthesized “in-house” which allowed fine-tuning of optimal AO target sequences at significantly reduced cost. Having ascertained an optimal AO sequence to skip exon 6 and 8 using 2OMe AOs, collaboration with AVI BioPharma (http://www.avibio.com/) presented the opportunity to initially study the potential of morpholino phosphorodiamidate oligomers (PMO) and later, the same compound with a covalently attached cell-penetrating peptide (PMO-Pep), for exon skipping. The low efficacy of PMOs to induce exon 6 and 8 skipping \textit{in vitro}, initially suggested that this was a poor choice of chemistry for application to exon skipping. However, subsequent successful experiments with the PMO-Pep indicated that the
morpholino chemistry could induce exon skipping, but that the use of a cell-penetrating peptide was necessary to induce efficient delivery into cultured cells. This correlated with parallel studies in our laboratory on the *mdx* mouse model where the PMOs had poor efficacy *in vitro*, although the addition of DNA “leashes” to the PMO was able to facilitate its uptake into the cell as a cationic lipoplex (Gebski et al., 2003). Both these results suggest that poor uptake of the PMO into the cell, rather than an inability to disrupt splicing, was the reason for its poor efficacy *in vitro*. Unlike negatively charged 2OMe AOs, PMOs are uncharged and as such, cannot be complexed with cationic liposomes for efficient delivery to the nucleus. As low level exon skipping could be detected, albeit at concentrations a thousand fold higher than for 2OMe AOs, a transport mechanism must exist although this has yet to be elucidated and could potentially be just passive diffusion of the PMO across the membrane. In contrast, the PMO-Peps induced very efficient skipping of exon 6 and 8 and significantly, were the only compounds to induce dystrophin protein in GRMD myotubes cultures. Although a higher concentration of PMO-Pep was necessary in comparison to the 2OMe AO, induction of corrected transcript persisted for longer and was the principle transcript present. This scenario would be preferable in a clinical setting, where persistence of correction would reduce the frequency of re-administration required to have a beneficial outcome as well as facilitate correction of a significantly higher proportion of functional dystrophin transcripts.

At the commencement of this PhD, only a small number of gene therapy studies had been undertaken with the GRMD model and were limited to local administration of plasmid dystrophin cDNA (Howell et al., 1998a) or mini-dystrophin constructs within adenoviral vectors (Howell et al., 1998b). These studies had generally poor outcomes, with low levels of dystrophin-positive fibres that were limited to near the site of injection (O'Hara et al., 2001). More recently, studies utilising either a cell replacement approach (Sampaolesi et al., 2006) or gene replacement using improved viral delivery vectors (Wang et al., 2007a, Wang et al., 2007b), have demonstrated greater success. The concept of using AOs though, to restore dystrophin expression in the GRMD model, had not been demonstrated until these studies were undertaken. Unlike the *mdx* mouse model which is readily available, access to the GRMD model was limited due to the closure in 2003 of the GRMD colony at
Murdoch University in Western Australia. Although collaboration with Professor Joe Kornegay at the University of Missouri, USA was initiated to facilitate in vivo testing, it was only possible to conduct pilot studies with a small number of animals. A more extensive study would be necessary for publication of the findings; however definitive proof-of-principle of an exon skipping approach to restore dystrophin expression in the GRMD animal was demonstrated.

In contrast to in vitro studies, both the PMO and PMO-Pep compounds were able to induce dystrophin protein expression, whilst no dystrophin could be detected in 20Me AO-treated animals. Given that the PMO-Pep induced dystrophin protein expression in vitro, it was not surprising that it would replicate this in vivo. However, the apparent contradiction with the PMO chemistry, which was very inefficient in culture and yet gave the greatest level of dystrophin expression in vivo, suggests that behavior of the PMO in the context of the three-dimensional structure of the muscle is significantly different to a monolayer system of differentiated myoblasts. Elucidating the reasons behind this difference would be an interesting study in itself, given that little is known about the nuclear uptake mechanism of a non-conjugated PMO and why it is so readily taken up into dystrophic muscle fibres. As restoration of dystrophin expression has been demonstrated in the mdx mouse following both intramuscular (Lu et al., 2003) and systemic administration (Lu et al., 2005) of the 20Me AO chemistry, the GRMD results were disappointing, particularly because this chemistry is currently being utilized in patient clinical studies. In the mdx model though, both PMO (Fletcher et al., 2006) and PMO-Pep (Fletcher et al., 2007) were superior to 20Me AOs of identical sequence in restoring dystrophin expression, which is consistent with the findings of the GRMD studies.

PMO and PMO-Pep treatment induced a dystrophin expression pattern similar to that observed using adenoviral gene therapy approaches (Gilbert et al., 2001), namely large numbers of dystrophin-positive fibres around the site of injection. These levels of expression were considerably less than recent studies using adeno-associated viral (AAV)-mediated dystrophin gene replacement where nearly all fibres expressed dystrophin
following intramuscular injection, (Wang et al., 2007b) although this is not surprising considering AAV vectors are extremely efficient at transducing skeletal muscle (Gregorevic et al., 2004, Wang et al., 2005). Significantly, an immunosuppression regimen was necessary to facilitate dystrophin expression from the viral construct, as initial studies of AAV-mediated transgene delivery to skeletal muscles in dogs demonstrated a strong T-cell mediated immune response to vector-associated proteins (Wang et al., 2007a). AO based approaches would not be expected to induce an immune response and so unlike viral delivery vectors, re-administration should be possible in both animal models and in patients, assuming there are no unexpected adverse reactions to a particular sequence, or the PMO backbone. Repeated administration of PMOs or PMO-Peps in the GRMD model would be expected to increase dystrophin protein levels; with systemic delivery preferable to intramuscular injections which would not be clinically feasible and could not address the entire skeletal muscle system. Having established the proof-of-principle that an AO based approach can be used to induce dystrophin expression in the GRMD model; there is now a platform to investigate the potential of alternative antisense chemistries or modifications that enhance stability uptake/delivery, as they become available. Similarly, the success of novel delivery systems, whether chemical or physical, could be ascertained through application to a larger animal model of DMD with a more clinically relevant phenotype.

The studies presented in Chapters 4 and 5 focus on the application of AO-based exon skipping to the human DMD condition. In an attempt to understand the key elements of splicing recognition, as well as provide potential therapeutic benefit, a study was undertaken within our laboratory to design AOs that could induce exon skipping in all of the exons in the dystrophin transcript (Wilton et al., 2007). As part of this overall study, work presented in Chapter 4 focused on the removal of exons that lay within the “minor deletion hotspot”, more specifically the removal of exons 3 to 9. Given that manipulation of splicing around this region could potentially restore the reading frame of approximately 7.5% of DMD patients, this study has clinical relevance as well as contributing to understanding of the exon skipping approach. Becker muscular dystrophy (BMD) patients with in-frame deletions in this region generally have phenotypes ranging from intermediate to severe, with low levels of dystrophin protein and relatively early age of onset (Beggs et
al., 1991), deletions with mild symptoms have also been reported (Saotome et al., 2001). This would indicate that this region, which contains two actin binding sites (Ervasti and Campbell, 1993), plays an important role in dystrophin function and stability. A report of a patient with an exon 3-9 in-frame deletion who’s clinical phenotype was so mild that he was not diagnosed until 65 years of age (Heald et al., 1994), was therefore of great interest. We hypothesized that induction of this larger deletion through AO-induced exon skipping, may be clinically preferable than inducing the minimal change necessary to restore the mRNA reading frame for DMD patients with mutations in this region. Thus the aim of the studies presented in Chapter 4 was to determine if it was possible to induce skipping of multiple exons spanning hundreds of kilobases of primary gene transcript, to produce a dystrophin transcript which mimicked those found in phenotypically mild BMD patients.

A previous study to induce large multiple exon skips in the central rod domain of the dystrophin gene was largely unsuccessful (Aartsma-Rus et al., 2006). Significantly though, only the outermost exons where targeted, whereas we hypothesized that if highly efficacious AOs could be designed, each individual exon could be targeted for simultaneous removal. From the GRMD studies, it was known that targeting of consensus splice sites alone may not be sufficient to induce exon skipping and so in silico prediction was used to predict ESE regions for targeting. It is assumed that the mechanism of action of AOs is to bind to or near ESE sequences so as to disrupt or alter binding of serine/arginine-rich (SR) proteins which are involved in exon recognition through the recruitment of components of the splicing machinery. However what is not evident, is which of the predicted SR protein binding sites are crucial for promotion of exon recognition, and hence targeting for exon removal. Analysis of the AO sequences for targeting exons 3 through to 9, indicated that no singular predicted ESE site is common to all and none of the AOs target only a single SR binding site. As the SR binding motifs are only 5 to 7 bp in length it is likely that in silico prediction overestimates the true number of actual ESE sites, especially as it does not take into account pre-mRNA secondary structure which is likely important for SR protein binding. However, if multiple ESEs throughout an exon can bind SR proteins there is the potential that blockage of one ESE could lead to another site being utilized. Indeed a recent study demonstrated that binding of specific SR
proteins occurs at multiple sites within exon 23 of the mdx mouse such that, although the mdx nonsense mutation partially disrupted a multi-site ESE, it does not promote exon skipping, presumably because other ESE sites can compensate (Buvoli et al., 2007). Nonsense mutations in exon 29 of BMD and X-linked dilated cardiomyopathy patients in contrast, have been shown to cause skipping of the in-frame exon 29 in a subset of transcripts that produce significant levels of near full-length dystrophin (Franz et al., 2000, Ginjaar et al., 2000). These mutations lay within predicted ESE sequences and in contrast to the mdx nonsense mutation, their disruption causes exon skipping in a proportion of dystrophin transcripts. Analysis of another nonsense mutation in exon 29, that produces a DMD phenotype, indicated that this mutation did not lie within an ESE site, so there is no exon skipping to bypass the nonsense mutation (Aartsma-Rus, unpublished observations). Thus it could be proposed that some ESE sites are more important for exon recognition than others, and that optimally designed AOs are targeting these regions rather than ESEs which are not critical for exon definition. This hypothesis may explain why AOs targeting different regions of the same exon results in varying exon skipping efficiencies, although consideration of the effect of pre-mRNA secondary structure on AO accessibility should not be dismissed. Whilst for the exons targeted in this study a single AO was sufficient to induce exon removal, for some dystrophin exons it is necessary to utilize multiple AOs that target distinctly separate sites within the same exon (Adams et al., 2007, Wilton et al., 2007). This supports the idea that multiple ESE sites may have the potential to recruit SR proteins and for exons where multiple sites must be targeted, it could suggest that more than one highly active ESE binding site exists. Another consideration is that AO binding may not just act to mask the SR protein binding site, but may also affect the conformation of the pre-mRNA transcript such that formation of the splicing complex involved in exon recognition is disrupted. Whilst the complexity of interaction between AOs, SR proteins and ESE sites means that there are no simple rules that can be applied to produce an optimal AO sequence, in silico prediction can help define potential regions of the exon for targeting for skipping. Further refinement of AO sequence through subtle variations in AO position and/or length, can then be employed to improve AO efficacy for potential clinical use.
Multiple exon skipping through targeting of each individual exon was initially tested on a smaller scale by targeted removal of exons 3-5 and 6-9. These regions were chosen as they both induce in-frame deletions and remove a similar number of exons (three versus four). Incidentally, in the Leiden DMD deletion mutation database there are eight reported cases of exon 3-5 deletions, with three reported as a DMD phenotype, four as BMD and one not stated (White and den Dunnen, 2006). This reported variation could potentially be due to a failure to clearly define the deletion, as older diagnostic tests by multiplex PCR did not usually include analysis of exon 2. There are no reported cases of patients with an exon 6-9 deletion. Removal of both multi-exon regions was efficient as demonstrated by relatively low AO concentrations inducing persistent exon skipping in vitro. Despite this success, application of the same AOs to induce removal of exons 3 to 9 resulted in transcripts of varying size, such that whilst transcripts missing exon 3-9 were present, they were not the dominant transcript. Indeed, one of the difficulties of assaying the true level of exon 3-9 skipping was the presence of exon 2 to 10 alternatively spliced endogenous transcripts. These transcripts have been identified in BMD/DMD patients in previous studies (Chelly et al., 1991) and have been observed frequently by us in myoblast cultures derived from non-DMD biopsies. While it cannot be conclusively determined if the exon 2 to 10 spliced transcripts were due to targeted exon skipping, there is evidence for an AO-mediated effect; the reduction of endogenous transcript missing exons 2-9, a reduction in the full-length transcript, and presence of intermediate transcripts that indicated removal of additional exons to the exon 3-5 or 6-9 subsets. The efficient removal of the smaller exon 3-5 and 6-9 subsets indicates that multiple exon skipping through targeting of each individual exon is feasible. It can only be speculated that by disrupting such a large number of exons in this region, completion of pre-mRNA splicing from exons 2 and 10 is impaired. This could be due somewhat to their large spatial distance as these exons lie ~374 kb apart on the pre-mRNA. Although these transcripts have been detected in the normal population, they are at a very low level (Chelly et al., 1990) which suggests this alternative splicing is an inefficient process. Whereas when exons are in close proximity, as in the case of the patient with the exon 3-9 genomic deletion, the splicing process can proceed efficiently despite the abnormal splicing of exon 2 to 10. Additionally, in patients with exon 3-7 genomic deletions who are expected to have a DMD phenotype, intermediate to severe BMD phenotypes have been reported (Koenig et al., 1989), thought
to be due to natural exon skipping to produce in-frame transcripts. Thus it would appear that the smaller the size of the region to be skipped, the higher the efficiency of the splicing process. Induction of an exon 3-9 deletion in patients with genomic deletions in this area could potentially be easier, although further studies in patient cell lines would be necessary to confirm this.

One concern of applying an exon skipping approach to DMD is the functionality of the internally deleted dystrophin protein that is induced. There are obvious benefits in designing an exon skipping strategy that mimicked a genotype found in clinically mild BMD patients. A “cocktail” of AOs that could potentially treat a variety of dystrophin mutations within a region, rather than a custom treatment for each patient, would also be preferable in a clinical setting. As such, the potential benefits of multiple exon skipping warrants further study in this area to develop strategies to overcome current limitations.

With mounting evidence for the success of the AO-mediated exon skipping approach in murine, canine and human models of DMD, there has been a recent push towards clinical trials of both PMO and 2OMeAO compounds. Initial clinical trials, whether Phase 0 or Phase I, are undertaken in small numbers of subjects (<20) and are designed to assess the safety, tolerability and pharmacokinetics of a medicine, and if possible, provide early evidence of effectiveness of the compound and determine if it behaves as expected from preclinical studies (U.S. Department of Health and Human Services, 2006). Until 2007, neither 2OMe nor PMO compounds had been applied in humans to induce exon skipping for treatment of DMD, although significantly, safety profiles had already been established for PMOs in trials for restenosis (Devi et al., 2005, Iversen et al., 2003) and Hepatitis C, where almost 250 patients had been treated with no adverse effects (http://www.avibio.com/devNeugene.html). In an attempt to demonstrate proof-of-concept of exon skipping in human tissue prior to clinical trials, studies in Chapter 5 utilized human tissue explants as an intermediate model between an in vitro cellular model and intramuscular administration in humans. Whilst an isolated human biopsy could not hope to be a true representation of the skeletal muscle system, it has significantly more
complexity than previous human exon skipping studies where a monolayer of myotubes derived from myoblast cultures was employed. To demonstrate exon skipping, muscle fragments were incubated in media containing either 20Me, PMO or PMO-Pep compounds in the case of non-dystrophic muscle, and 20Me alone for DMD patient tissue. In the normal human tissue, the morpholino compounds induced the highest levels of exon skipping, consistent with in vivo studies in the GRMD and mdx mouse model.

Unfortunately only the 20Me chemistry was available to address the particular DMD mutation in the patient tissue; however despite using arguably the weaker of the two AO chemistries extensively evaluated to date, corrected dystrophin transcript could be detected for up to at least two weeks. Considering that morpholino compounds were more efficacious in non-DMD muscle, it was surmised that exon skipping in DMD patient tissue could be improved further with this chemistry. This study was the first evidence that dystrophin exon skipping can be undertaken in human muscle tissue, albeit in an ex vivo environment, and increases confidence that this approach will be successful when applied to DMD patients in clinical trials.

It has been twenty years since an absence of a functional dystrophin protein was determined to be the cause of DMD (Hoffman et al., 1987). However, sadly to date, there is still no treatment available that addresses the principle dystrophin genetic defect. That is not to say there has not been progress. Preservation of muscle function with regimens of corticosteroid, spinal corrective surgery and night-assisted ventilation to reduce the burden on the diaphragm muscle, have increased life expectancy from early twenties to well into the fourth decade of life (Eagle et al., 2007). However, if a significant improvement in quality of life is to be achieved then genetic therapy will be required to restore the normal function of the muscle.

So what is the future for DMD patients? Some of the greatest successes in animal models have been with the use of viral vectors to deliver either partial dystrophin cDNAs (that contain the minimal elements required to have functional benefit) (Gregorevic et al., 2006, Wang et al., 2007b) or to express small nuclear RNAs which can target the
dystrophin pre-mRNA to induce exon skipping (Goyenvalle et al., 2004). However, before these studies can be translation to human application, potent immune responses to the viral proteins will need to be addressed. Clinical trials in myoblast transplantation in the nineties proved unsuccessful mainly due to poor survival of donor myoblasts (Skuk and Tremblay, 2000) but with recent advances in the understanding of stem cells, it may be feasible to isolate patient non-myogenic stem cells, such as mesoangioblasts (Sampaolesi et al., 2006), and proliferate and modify them ex vivo before delivery to the patient. For the subset of DMD patients with nonsense mutations, recent demonstration of dystrophin expression in human cell models and in the mdx mouse following administration of the small drug PTC124 (Welch et al., 2007), as well as successful Phase I clinical trials in healthy adult volunteers (Hirawat et al., 2007), offers hope for a treatment in the near future.

AO-mediated exon skipping to address DMD has moved relatively rapidly from its first application to DMD in 1996 (Pramono et al., 1996) to the commencement of clinical trials in 2006/2007. Phase I clinical trials utilizing the 2OMe chemistry directed against exon 51, have been undertaken by the Leiden DMD group led by Gert-Jan van Ommen and Judith Van Deutekom with no reported adverse effects and dystrophin-positive fibres around the injection site (Van Deutekom, 2007). Patients are currently being recruited by the UK MDEX consortium for an independent parallel Phase I study of local administration of a PMO directed against exon 51(Arechavala-Gomeza et al., 2007) into the extensor digitorum brevis. With the success of the PMO chemistry in mdx and GRMD animal models, as well as demonstration that the PMO can induce exon skipping in human explants, there is great hope for these trials. Whilst intramuscular injection is sufficient to demonstrate proof-of-principle of exon skipping, repeat administration would not be practical in a clinical setting nor would it address the entire skeletal muscle system. A systemic approach to deliver AOs to all muscles, including the heart, will be necessary to ameliorate the dystrophic condition. In designing an exon skipping approach in a clinically relevant, larger DMD animal model it is hoped it is hoped that this will facilitate the testing of any new delivery systems or AO modifications that may arise in the future. Although an exon skipping approach will not address all DMD mutations, ~90% of DMD patients could theoretically benefit (Aartsma-Rus et al., 2004). Equally, whilst exon skipping will never
be a cure; restoration of dystrophin expression would be expected to convey a significant improvement in the quality of life for DMD boys. A multi-faceted genetic approach including AO-mediated exon skipping, along with supplements that address dystrophin pathology and improvements in physical care, is how I personally foresee the therapeutic future for DMD patients.
6.2 References


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