

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

Chalermchai Mitrpant^{1,2,3}, Sue Fletcher¹ and Steve D. Wilton*¹

¹Molecular Genetic Therapy Group, Centre for Neuromuscular and Neurological Diseases, University of Western Australia, QEII Medical Centre, Nedlands, Western Australia, Australia; ²Centre for Human Genetics, Edith Cowan University, 100 Joondalup Drive, Perth WA 6027, Australia; ³Department of Biochemistry, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

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

Keywords: Antisense oligonucleotides, exon skipping, duchenne muscular dystrophy, morpholino, clinical trials, dystrophin, personalized medicine, pre-mRNA splicing.

INTRODUCTION

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

It is no longer appropriate to consider single gene disorders as "simple", particularly since it is apparent that different lesions in a particular gene can result in a variety of clinically distinguishable conditions, with either recessive or dominant modes of transmission. Although the $\Delta F508$ mutation defect accounts for about 75% of cystic fibrosis cases [2, 3], over 1,000 different mutations have been reported and the clinical presentation can vary considerably from severe, with extensive lung involvement, to a very mild phenotype with reduced fertility as the predominant symptom. Similarly, different mutations in the huge dystrophin gene can lead to the allelic conditions, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), and X-linked dilated cardiomyopathy [4].

Restoration of expression of a single component to address a missing or defective gene product is the basis of gene replacement (viral and non-viral systems) or cell-based therapies. Although great advances are being made in the treatment of some conditions, for example, gene replacement in children with X-linked severe combined immune deficiency (SCID) [5, 6], there have also been serious adverse events and progress has not been as rapid as anticipated [7]. Challenges to gene and cell replacement therapy for DMD arise from the size of the gene product and/or nature of the gene expres-

sion. Dystrophin, the affected gene product in DMD, is encoded by the largest known gene and is expressed as multiple isoforms in different tissues, with the 427 kDa skeletal muscle protein produced at low amounts in about 30% of the human body mass [8].

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

DUCHENNE AND BECKER MUSCULAR DYSTROPHY

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

*Address correspondence to this author at the Centre for Neuromuscular and Neurological Disorders Australian Neuromuscular Research Institute 4th Floor "A" Block, QE II Medical Centre Nedlands 6009 Western Australia; Tel: +61 8 93463967; Fax: +61 8 93463487; Mob: (+61) 0417982365; E-mail: swilton@cyllene.uwa.edu.au

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

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

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

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

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

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

Dystrophin is thought to act much like a shock absorber linking the actin cytoskeleton to a complex of proteins embedded in the

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

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

NATURAL PRECEDENTS FOR EXON EXCLUSION DURING DYSTROPHIN PROCESSING

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

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

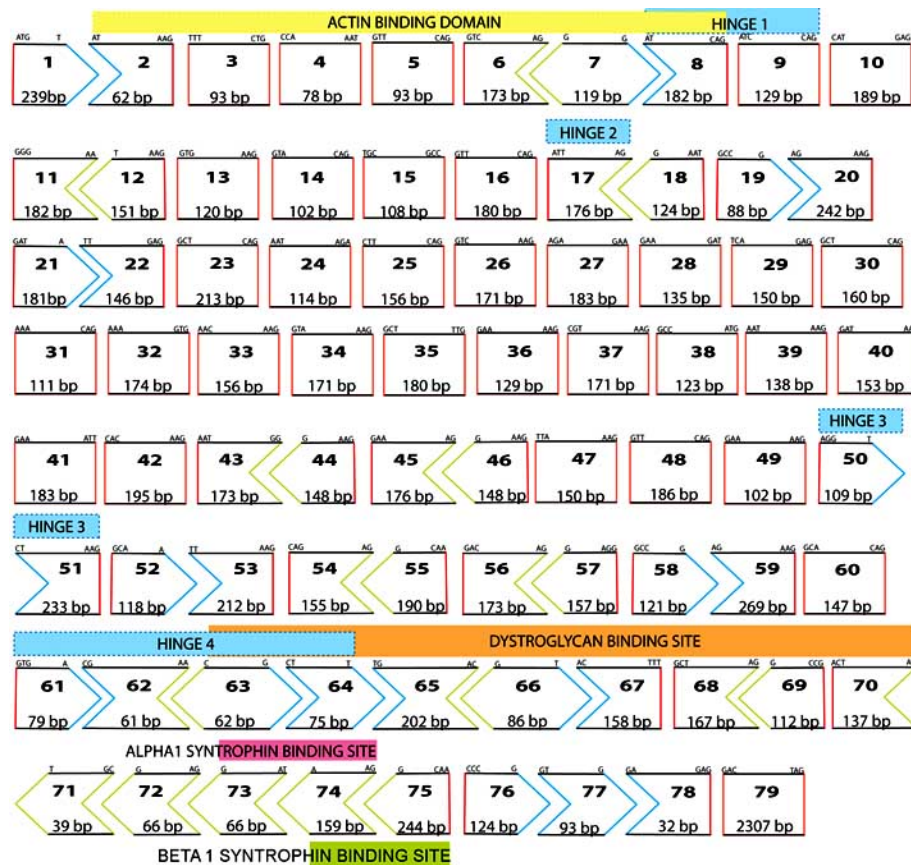


Fig. (1). Structure of the dystrophin gene transcript indicating the reading frame and major functional domains. Boxes represent in-frame exons whereas interlocking forward and reverse arrows and notches indicate codons spanning the exon:exon junction. Junction codon sequences are shown above the exons.

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

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

ANTISENSE OLIGOMERS AND MODES OF ACTION

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

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

EARLY SPLICE INTERVENTION STUDIES

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

ANIMAL MODELS

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

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

AO DESIGN AND TARGET SELECTION

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

exon 23 skipping, whereas a 20mer directed at the acceptor site was found to be ineffective [69]. Subsequent refinements in AOs targeting the dystrophin exon 23 donor splice site were reported and found to further enhance the capacity of the AO to induce exon 23 skipping [70]. Additional AO refinement at the acceptor splice site consistently failed to induce any detectable skipping [70].

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

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

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

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

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

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

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

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

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

Some dystrophin exons were difficult to dislodge from the mature dystrophin mRNA, and despite designing AOs across most of the exon, with either no or only very low levels of skipping being induced after transfection with high AO:lipoplex concentrations. Exon 20 was one such example, in which over 20 different AOs

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

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

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

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

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

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

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

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

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

It is not realistic to consider using cationic liposome preparations for repeated systemic delivery, based upon cost and more importantly, potential toxicity. The pluronic block co-polymer F127 was shown to enhance uptake of 2OMe AOs [106, 107] and Wells *et al.* [108] showed enhanced AO delivery in the mouse using electroporation. However, it is possible that systemic delivery may not be as great a challenge for PMOs as first anticipated. *In vivo* administration of a PMO was undertaken by injecting a cationic lipoplex composed of a PMO annealed to a sense strand leash, (1-5 µg) directed to the donor splice site of mouse dystrophin exon 23. As anticipated from *in vitro* studies, substantial dystrophin exon skip-

ping was detected at the RNA and protein levels [100]. Immunofluorescence indicated that the induced dystrophin was correctly localized and the sarcolemmal complex was re-established. What was unexpected was that similar levels of exon skipping were induced after administration of an equivalent amount of PMO that was not annealed to the leash. It quickly became apparent that *in vivo* PMO uptake was much more efficient than anticipated from *in vitro* studies. Systemic studies followed and dystrophin expression could be detected in all tissues examined, except the heart [109, 110]. Further advances in PMO delivery came with conjugation to cell penetrating peptides, and even more substantial dystrophin expression was induced, using even lower doses of the PMO [111]. However, to date, the heart remains resistant to AO-induced exon skipping, prompting additional studies using different peptide tags and dosage regimens.

ALTERNATIVE EXON SKIPPING STRATEGIES

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

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

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

CLINICAL TRIALS

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

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

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

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

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

FUTURE CHALLENGES

There is considerable optimism that AO-induced exon skipping may substantially reduce the progression and symptoms of DMD. However, a number of major challenges lie ahead. First, the classification of exon skipping as a gene therapy is regarded differently by regulatory agencies in different countries. We propose that the

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

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

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

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

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

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

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

Several PMOs of different sequences have been tested in animals, including mouse, rat, dog, and non-human primate for general toxicity and side-effects that may be associated with the backbone chemistry [105]. While safety studies of oligomers designed for viral gene suppression have been undertaken in normal human volunteers, similar safety trials cannot be considered for testing AVI-4658, a PMO designed to excise dystrophin exon 51. Evaluating AVI-4658 in normal human volunteers must be regarded as unethical due to unacceptable risks to the participants. If this compound works exactly as predicted, exon 51 would be removed from the normal dystrophin gene transcript, disrupting the reading frame and potentially inducing DMD. As shown in Fig. (2), the consequences of exon 51 skipping will vary extensively between normal individuals and different DMD patients. In what should be a most amenable mutation (DMD $\Delta 50$), AVI-4658 would restore the reading frame around this single exon deletion, and allow synthesis of a dystrophin of near-normal length and function. The nature of induced dystrophin isoform will depend upon the extent of the primary gene

deletion, hence potential benefits of exon skipping would be compromised by larger gene deletions; such as DMD $\Delta 30-50$ and $\Delta 13-50$. Thus, there is a broad spectrum of potential consequences of administering AVI-4658 to different individuals, ranging from no restoration of the reading frame in DMD patients with non-responsive mutations, induction of dystrophin isoforms of variable function depending upon the primary gene lesion, to inducing DMD in a normal individual by disrupting the dystrophin reading frame.

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

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

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

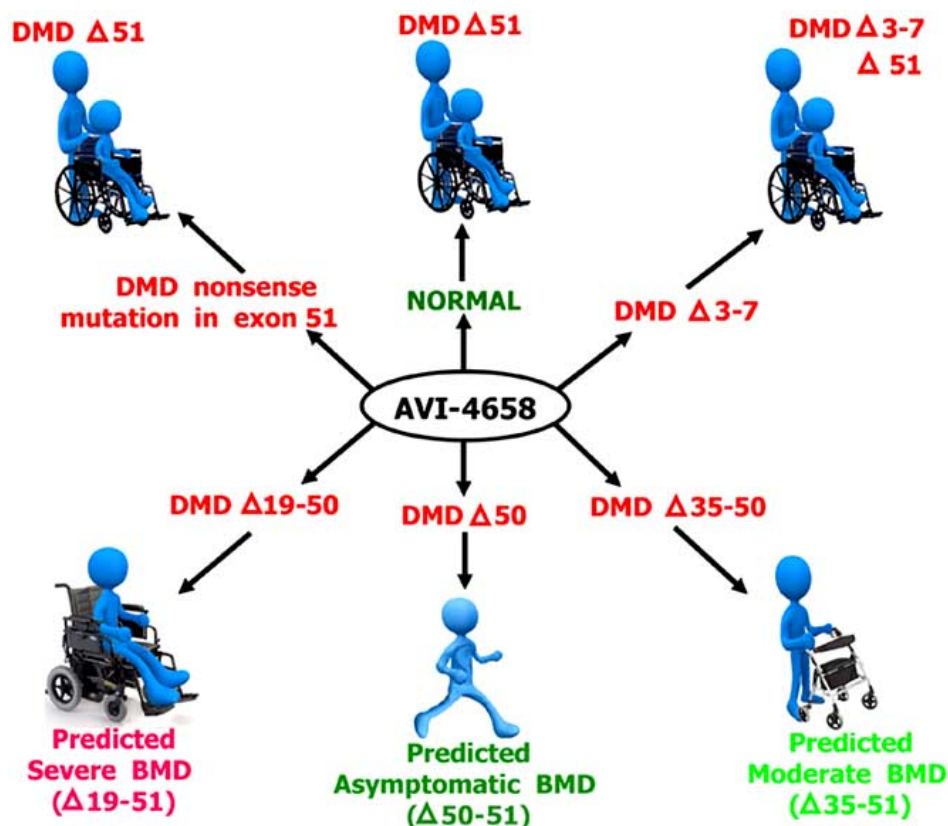


Fig. (2). Potential consequences of AO induced exon 51 skipping in individuals with different dystrophin genes. Δn indicates deleted exons before and after treatment. $\Delta 19-51$, $\Delta 35-51$, and $\Delta 50-51$ are all in-frame transcripts and should lead to various BMD phenotypes.

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

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

ACKNOWLEDGEMENTS

We wish to acknowledge support from the following funding bodies: National Institutes of Health, Muscular Dystrophy Association of the United States, Neuromuscular Foundation of Western Australia, Charley's Fund and the Medical Health Research Infrastructure Fund of Western Australia. CM was supported by a scholarship from the Faculty of Medicine, Siriraj Hospital, Mahidol University.

ABBREVIATIONS

AO	=	Antisense oligomer
BMD	=	Becker muscular dystrophy
cDNA	=	Complementary DNA
DMD	=	Duchenne muscular dystrophy
DNA	=	Deoxyribonucleic acid
ENAs	=	4'-C-ethylene bridge nucleic acids
ESE	=	Exon splicing enhancer
GRMD	=	Golden retriever muscular dystrophy
kDa	=	Kilodalton
LNAs	=	Locked nucleic acids
MOE	=	2'-O-methoxy-ethoxy AO
NMD	=	Nonsense mediated decay
ODN	=	Oligodeoxynucleotide
PMO	=	Phosphorodiamidate Morpholino Oligomer
PNAs	=	Peptide nucleic acids
pre-mRNA	=	Precursor messenger ribonucleic acid
RNA	=	Ribonucleic acid
SCID	=	Severe combined immunodeficiency
2OMe	=	2'-O-methyl modified on a phosphorothioate backbone

REFERENCES

[1] Dodge, J. A.; Lewis, P. A.; Stanton, M.; Wilsher, J. Cystic fibrosis mortality and survival in the UK: 1947-2003. *Eur. Respir. J.* **2007**, *29*, 522-526.
 [2] McIntosh, I.; Lorenzo, M. L.; Brock, D. J. Frequency of delta F508 mutation on cystic fibrosis chromosomes in UK. *Lancet* **1989**, *2*, 1404-1405.

[3] Lemna, W. K.; Feldman, G. L.; Kerem, B.; Fernbach, S. D.; Zevkovich, E. P.; O'Brien, W. E.; Riordan, J. R.; Collins, F. S.; Tsui, L. C.; Beaudet, A. L. Mutation analysis for heterozygote detection and the prenatal diagnosis of cystic fibrosis. *N. Engl. J. Med.* **1990**, *322*, 291-296.
 [4] Ferlini, A.; Sewry, C.; Melis, M. A.; Mateddu, A.; Muntoni, F. X-linked dilated cardiomyopathy and the dystrophin gene. *Neuromuscul. Disord.* **1999**, *9*, 339-346.
 [5] Hacein-Bey, S.; Gross, F.; Nusbaum, P.; Hue, C.; Hamel, Y.; Fischer, A.; Cavazzana-Calvo, M. Optimization of retroviral gene transfer protocol to maintain the lymphoid potential of progenitor cells. *Hum. Gene Ther.* **2001**, *12*, 291-301.
 [6] Cavazzana-Calvo, M.; Hacein-Bey, S.; de Saint Basile, G.; Gross, F.; Yvon, E.; Nusbaum, P.; Selz, F.; Hue, C.; Certain, S.; Casanova, J. L.; Bousso, P.; Deist, F. L.; Fischer, A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **2000**, *288*, 669-672.
 [7] Hacein-Bey-Abina, S.; von Kalle, C.; Schmidt, M.; Le Deist, F.; Wulffraat, N.; McIntyre, E.; Radford, I.; Villeval, J. L.; Fraser, C. C.; Cavazzana-Calvo, M.; Fischer, A. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **2003**, *348*, 255-256.
 [8] Janssen, I.; Heymsfield, S. B.; Wang, Z. M.; Ross, R. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *J. Appl. Physiol.* **2000**, *89*, 81-88.
 [9] Mattei, E.; Corbi, N.; Di Certo, M. G.; Strimpakos, G.; Severini, C.; Onori, A.; Desantis, A.; Libri, V.; Buontempo, S.; Floridi, A.; Fanciulli, M.; Baban, D.; Davies, K. E.; Passananti, C. Utrrophin up-regulation by an artificial transcription factor in transgenic mice. *PLoS One* **2007**, *2*, e774.
 [10] Welch, E. M.; Barton, E. R.; Zhuo, J.; Tomizawa, Y.; Friesen, W. J.; Trifillis, P.; Paushkin, S.; Patel, M.; Trotta, C. R.; Hwang, S.; Wilde, R. G.; Karp, G.; Takasugi, J.; Chen, G.; Jones, S.; Ren, H.; Moon, Y. C.; Corson, D.; Turpoff, A. A.; Campbell, J. A.; Conn, M. M.; Khan, A.; Almstead, N. G.; Hedrick, J.; Mollin, A.; Risher, N.; Weetall, M.; Yeh, S.; Branstrom, A. A.; Colacic, J. M.; Babiak, J.; Ju, W. D.; Hirawat, S.; Northcutt, V. J.; Miller, L. L.; Spatric, P.; He, F.; Kawana, M.; Feng, H.; Jacobson, A.; Peltz, S. W.; Sweeney, H. L. PTC124 targets genetic disorders caused by nonsense mutations. *Nature* **2007**, *447*, 87-91.
 [11] Minetti, G. C.; Colussi, C.; Adami, R.; Serra, C.; Mozzetta, C.; Parente, V.; Fortuni, S.; Straino, S.; Sampaolesi, M.; Di Padova, M.; Illi, B.; Gallinari, P.; Steinkuhler, C.; Capogrossi, M. C.; Sartorelli, V.; Bottinelli, R.; Gaetano, C.; Puri, P. L. Functional and morphological recovery of dystrophic muscles in mice treated with deacetylase inhibitors. *Nat. Med.* **2006**, *12*, 1147-1150.
 [12] Bakker, E.; Veenema, H.; Den Dunnen, J. T.; van Broeckhoven, C.; Grootsholten, P. M.; Bonten, E. J.; van Ommen, G. J.; Pearson, P. L. Germinal mosaicism increases the recurrence risk for 'new' Duchenne muscular dystrophy mutations. *J. Med. Genet.* **1989**, *26*, 553-559.
 [13] Kunkel, L. M.; Beggs, A. H.; Hoffman, E. P. Molecular genetics of Duchenne and Becker muscular dystrophy: emphasis on improved diagnosis. *Clin. Chem.* **1989**, *35*, B21-24.
 [14] Bushby, K.; Bourke, J.; Bullock, R.; Eagle, M.; Gibson, M.; Quinby, J. The multidisciplinary management of Duchenne muscular dystrophy. *Curr. Paediatr.* **2005**, *15*, 292-300.
 [15] Prior, T. W.; Bridgeman, S. J. Experience and strategy for the molecular testing of Duchenne muscular dystrophy. *J. Mol. Diagn.* **2005**, *7*, 317-326.
 [16] Heald, A.; Anderson, L. V.; Bushby, K. M.; Shaw, P. J. Becker muscular dystrophy with onset after 60 years. *Neurology* **1994**, *44*, 2388-2390.
 [17] Schwartz, M.; Duno, M.; Palle, A. L.; Krag, T.; Vissing, J. Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum. Mutat.* **2007**, *28*, 205.
 [18] Hoffman, E. P.; Fischbeck, K. H.; Brown, R. H.; Johnson, M.; Medori, R.; Loike, J. D.; Harris, J. B.; Waterston, R.; Brooke, M.; Specht, L.; Kupsky, W.; Chamberlain, J.; Caskey, C. T.; Shapiro, F.; Kunkel, L. M. Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy. *N. Engl. J. Med.* **1988**, *318*, 1363-1368.
 [19] Den Dunnen, J. T.; Grootsholten, P. M.; Bakker, E.; Blonden, L. A.; Ginjaar, H. B.; Wapenaar, M. C.; van Paassen, H. M.; van Broeckhoven, C.; Pearson, P. L.; van Ommen, G. J. Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. *Am. J. Hum. Genet.* **1989**, *45*, 835-847.
 [20] White, S. J.; Aartsma-Rus, A.; Flanigan, K. M.; Weiss, R. B.; Kneppers, A. L.; Lalic, T.; Janson, A. A.; Ginjaar, H. B.; Breuning, M. H.; den Dunnen, J. T. Duplications in the DMD gene. *Hum. Mutat.* **2006**, *27*, 938-945.
 [21] Aartsma-Rus, A.; Van Deutekom, J. C.; Fokkema, I. F.; Van Ommen, G. J.; Den Dunnen, J. T. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* **2006**, *34*, 135-144.
 [22] Taylor, P. J.; Maroulis, S.; Mullan, G. L.; Pedersen, R. L.; Baumli, A.; Elakis, G.; Piras, S.; Walsh, C.; Prosper-Gutierrez, B.; De La Puente-Alonso, F.; Bell, C. G.; Mowat, D. R.; Johnston, H. M.; Buckley, M. F. Measurement of the clinical utility of a combined mutation detection protocol in carriers of Duchenne and Becker muscular dystrophy. *J. Med. Genet.* **2007**, *44*, 368-372.
 [23] Emery, A. E. Muscular dystrophy into the new millennium. *Neuromuscul. Disord.* **2002**, *12*, 343-349.
 [24] Emery, A. E. The muscular dystrophies. *Lancet* **2002**, *359*, 687-695.

- [25] Blake, D. J.; Weir, A.; Newey, S. E.; Davies, K. E. Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol. Rev.* **2002**, *82*, 291-329.
- [26] Foster, K.; Foster, H.; Dickson, J. G. Gene therapy progress and prospects: Duchenne muscular dystrophy. *Gene Ther.* **2006**, *13*, 1677-1685.
- [27] Odom, G. L.; Gregorevic, P.; Chamberlain, J. S. Viral-mediated gene therapy for the muscular dystrophies: successes, limitations and recent advances. *Biochim. Biophys. Acta* **2007**, *1772*, 243-262.
- [28] Corrado, K.; Mills, P. L.; Chamberlain, J. S. Deletion analysis of the dystrophin-actin binding domain. *FEBS Lett.* **1994**, *344*, 255-260.
- [29] Norwood, F. L.; Sutherland-Smith, A. J.; Keep, N. H.; Kendrick-Jones, J. The structure of the N-terminal actin-binding domain of human dystrophin and how mutations in this domain may cause Duchenne or Becker muscular dystrophy. *Structure* **2000**, *8*, 481-491.
- [30] Cai, H.; Erdman, R. A.; Zweier, L.; Chen, J.; Shaw, J. H. t.; Baylor, K. A.; Stecker, M. M.; Carey, D. J.; Chan, Y. M. The sarcoglycan complex in Schwann cells and its role in myelin stability. *Exp. Neurol.* **2007**, *205*, 257-269.
- [31] Durbeek, M.; Jung, D.; Hjalt, T.; Campbell, K. P.; Ekblom, P. Transient expression of Dp140, a product of the Duchenne muscular dystrophy locus, during kidney tubulogenesis. *Dev. Biol.* **1997**, *181*, 156-167.
- [32] Marquez, F. G.; Cisneros, B.; Garcia, F.; Ceja, V.; Velazquez, F.; Depardon, F.; Cervantes, L.; Rendon, A.; Mornet, D.; Rosas-vargas, H.; Mustre, M.; Montanez, C. Differential expression and subcellular distribution of dystrophin Dp71 isoforms during differentiation process. *Neuroscience* **2003**, *118*, 957-966.
- [33] Passos-Bueno, M. R.; Vainzof, M.; Marie, S. K.; Zatz, M. Half the dystrophin gene is apparently enough for a mild clinical course: confirmation of its potential use for gene therapy. *Hum. Mol. Genet.* **1994**, *3*, 919-922.
- [34] England, S. B.; Nicholson, L. V.; Johnson, M. A.; Forrest, S. M.; Love, D. R.; Zubrzycka-Gaarn, E. E.; Bulman, D. E.; Harris, J. B.; Davies, K. E. Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* **1990**, *343*, 180-182.
- [35] Sicinski, P.; Geng, Y.; Ryder-Cook, A. S.; Barnard, E. A.; Darlison, M. G.; Barnard, P. J. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* **1989**, *244*, 1578-1580.
- [36] Wilton, S. D.; Dye, D. E.; Blechyniden, L. M.; Laing, N. G. Revertant fibres: a possible genetic therapy for Duchenne muscular dystrophy? *Neuromuscul. Disord.* **1997**, *7*, 329-335.
- [37] Kornegay, J. N.; Tuler, S. M.; Miller, D. M.; Levesque, D. C. Muscular dystrophy in a litter of golden retriever dogs. *Muscle Nerve* **1988**, *11*, 1056-1064.
- [38] Lanfossi, M.; Cozzi, F.; Bugini, D.; Colombo, S.; Scarpa, P.; Morandi, L.; Galbiati, S.; Cornelio, F.; Pozza, O.; Mora, M. Development of muscle pathology in canine X-linked muscular dystrophy. I. Delayed postnatal maturation of affected and normal muscle as revealed by myosin isoform analysis and utrophin expression. *Acta Neuropathol. (Berl.)* **1999**, *97*, 127-138.
- [39] Sharp, N. J.; Kornegay, J. N.; Van Camp, S. D.; Herbstreith, M. H.; Secore, S. L.; Kettle, S.; Hung, W. Y.; Constantinou, C. D.; Dykstra, M. J.; Roses, A. D.; Bartlett, R. J. An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy. *Genomics* **1992**, *13*, 115-121.
- [40] Wallgren-Pettersson, C.; Jasani, B.; Rosser, L. G.; Lazarou, L. P.; Nicholson, L. V.; Clarke, A. Immunohistological evidence for second or somatic mutations as the underlying cause of dystrophin expression by isolated fibres in Xp21 muscular dystrophy of Duchenne-type severity. *J. Neurol. Sci.* **1993**, *118*, 56-63.
- [41] Nicholson, L. V.; Johnson, M. A.; Bushby, K. M.; Gardner-Medwin, D. Functional significance of dystrophin positive fibres in Duchenne muscular dystrophy. *Arch. Dis. Child.* **1993**, *68*, 632-636.
- [42] Fanin, M.; Danieli, G. A.; Vitiello, L.; Senter, L.; Angelini, C. Prevalence of dystrophin-positive fibers in 85 Duchenne muscular dystrophy patients. *Neuromuscul. Disord.* **1992**, *2*, 41-45.
- [43] Sherratt, T. G.; Vulliamy, T.; Dubowitz, V.; Sewry, C. A.; Strong, P. N. Exon skipping and translation in patients with frameshift deletions in the dystrophin gene. *Am. J. Hum. Genet.* **1993**, *53*, 1007-1015.
- [44] Schatzberg, S. J.; Olby, N. J.; Breen, M.; Anderson, L. V.; Langford, C. F.; Dickens, H. F.; Wilton, S. D.; Zeiss, C. J.; Binns, M. M.; Kornegay, J. N.; Morris, G. E.; Sharp, N. J. Molecular analysis of a spontaneous dystrophin 'knockout' dog. *Neuromuscul. Disord.* **1999**, *9*, 289-295.
- [45] Thanh, L. T.; Nguyen, T. M.; Helliwell, T. R.; Morris, G. E. Characterization of revertant muscle fibers in Duchenne muscular dystrophy, using exon-specific monoclonal antibodies against dystrophin. *Am. J. Hum. Genet.* **1995**, *56*, 725-731.
- [46] Lu, Q. L.; Morris, G. E.; Wilton, S. D.; Ly, T.; Artem'yeva, O. V.; Strong, P.; Partridge, T. A. Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J. Cell. Biol.* **2000**, *148*, 985-996.
- [47] Klein, C. J.; Covert, D. D.; Bulman, D. E.; Ray, P. N.; Mendell, J. R.; Burghes, A. H. Somatic reversion/suppression in Duchenne muscular dystrophy (DMD): evidence supporting a frame-restoring mechanism in rare dystrophin-positive fibers. *Am. J. Hum. Genet.* **1992**, *50*, 950-959.
- [48] Wilton, S. D.; Johnsen, R. D.; Pedretti, J. R.; Laing, N. G. Two distinct mutations in a single dystrophin gene: identification of an altered splice-site as the primary Becker muscular dystrophy mutation. *Am. J. Med. Genet.* **1993**, *46*, 563-569.
- [49] Tuffery-Giraud, S.; Saquet, C.; Thorel, D.; Disset, A.; Rivier, F.; Malcolm, S.; Claustres, M. Mutation spectrum leading to an attenuated phenotype in dystrophinopathies. *Eur. J. Hum. Genet.* **2005**, *13*, 1254-1260.
- [50] Ginjaar, I. B.; Kneppers, A. L.; v d Meulen, J. D.; Anderson, L. V.; Bremmer-Bout, M.; van Deutekom, J. C.; Weegenaar, J.; den Dunnen, J. T.; Bakker, E. Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. *Eur. J. Hum. Genet.* **2000**, *8*, 793-796.
- [51] Monaco, A. P.; Bertelson, C. J.; Liechti-Gallati, S.; Moser, H.; Kunkel, L. M. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* **1988**, *2*, 90-95.
- [52] Yan, J.; Feng, J.; Buzin, C. H.; Scaringe, W.; Liu, Q.; Mendell, J. R.; den Dunnen, J.; Sommer, S. S. Three-tiered noninvasive diagnosis in 96% of patients with Duchenne muscular dystrophy (DMD). *Hum. Mutat.* **2004**, *23*, 203-204.
- [53] Stockley, T. L.; Akber, S.; Bulgin, N.; Ray, P. N. Strategy for comprehensive molecular testing for Duchenne and Becker muscular dystrophies. *Genet. Test.* **2006**, *10*, 229-243.
- [54] Gurvich, O. L.; Tuohy, T. M.; Howard, M. T.; Finkel, R. S.; Medne, L.; Anderson, C. B.; Weiss, R. B.; Wilton, S. D.; Flanigan, K. M. DMD pseudo-exon mutations: splicing efficiency, phenotype, and potential therapy. *Ann. Neurol.* **2008**, *63*, 81-89.
- [55] Stephenson, M. L.; Zamecnik, P. C. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 285-288.
- [56] Zamecnik, P. C.; Stephenson, M. L. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 280-284.
- [57] The Vitravene Study Group. A randomized controlled clinical trial of intravitreal fomivirsen for treatment of newly diagnosed peripheral cytomegalovirus retinitis in patients with aids. *Am. J. Ophthalmol.* **2002**, *133*, 467-474.
- [58] Grishok, A.; Sinskey, J. L.; Sharp, P. A. Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. *Genes Dev.* **2005**, *19*, 683-696.
- [59] Houmar, N. M.; Mainville, J. L.; Bonin, C. P.; Huang, S.; Luethy, M. H.; Malvar, T. M. High-lysine corn generated by endospem-specific suppression of lysine catabolism using RNAi. *Plant Biotechnol. J.* **2007**, *5*, 605-614.
- [60] Schmitz, G. Drug evaluation: OXG-011, a clusterin-inhibiting antisense oligonucleotide. *Curr. Opin. Mol. Ther.* **2006**, *8*, 547-554.
- [61] Morris, K. V. VRX-496 (VIRxSYS). *Curr. Opin. Investig. Drugs* **2005**, *6*, 209-215.
- [62] Dominski, Z.; Kole, R. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8673-8677.
- [63] Pramono, Z. A.; Takeshima, Y.; Alimsardjono, H.; Ishii, A.; Takeda, S.; Matsuo, M. Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. *Biochem. Biophys. Res. Commun.* **1996**, *226*, 445-449.
- [64] Matsuo, M.; Masumura, T.; Nakajima, T.; Kitoh, Y.; Takumi, T.; Nishio, H.; Koga, J.; Nakamura, H. A very small frame-shifting deletion within exon 19 of the Duchenne muscular dystrophy gene. *Biochem. Biophys. Res. Commun.* **1990**, *170*, 963-967.
- [65] Matsuo, M.; Masumura, T.; Nishio, H.; Nakajima, T.; Kitoh, Y.; Takumi, T.; Koga, J.; Nakamura, H. Exon skipping during splicing of dystrophin mRNA precursor due to an intraxon deletion in the dystrophin gene of Duchenne muscular dystrophy kobe. *J. Clin. Invest.* **1991**, *87*, 2127-2131.
- [66] Stedman, H. H.; Sweeney, H. L.; Shrager, J. B.; Maguire, H. C.; Panettieri, R. A.; Petrof, B.; Narusawa, M.; Leferovich, J. M.; Sladky, J. T.; Kelly, A. M. The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* **1991**, *352*, 536-539.
- [67] Deconinck, N.; Rafael, J. A.; Beckers-Bleux, G.; Kahn, D.; Deconinck, A. E.; Davies, K. E.; Gillis, J. M. Consequences of the combined deficiency in dystrophin and utrophin on the mechanical properties and myosin composition of some limb and respiratory muscles of the mouse. *Neuromuscul. Disord.* **1998**, *8*, 362-370.
- [68] Duncley, M. G.; Manoharan, M.; Villiet, P.; Eperon, I. C.; Dickson, G. Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligonucleotides. *Hum. Mol. Genet.* **1998**, *7*, 1083-1090.
- [69] Wilton, S. D.; Lloyd, F.; Carville, K.; Fletcher, S.; Honeyman, K.; Agrawal, S.; Kole, R. Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides. *Neuromuscul. Disord.* **1999**, *9*, 330-338.
- [70] Mann, C. J.; Honeyman, K.; McCloy, G.; Fletcher, S.; Wilton, S. D. Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. *J. Gene Med.* **2002**, *4*, 644-654.
- [71] van Deutekom, J. C.; Bremmer-Bout, M.; Janson, A. A.; Ginjaar, I. B.; Baas, F.; den Dunnen, J. T.; van Ommen, G. J. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum. Mol. Genet.* **2001**, *10*, 1547-1554.
- [72] Aartsma-Rus, A.; Kaman, W. E.; Bremmer-Bout, M.; Janson, A. A.; den Dunnen, J. T.; van Ommen, G. J.; van Deutekom, J. C. Comparative analysis

- of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. *Gene Ther.* **2004**, *11*, 1391-1398.
- [73] Aartsma-Rus, A.; Janson, A. A.; Kaman, W. E.; Bremmer-Bout, M.; van Ommen, G. J.; den Dunnen, J. T.; van Deutekom, J. C. Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. *Am. J. Hum. Genet.* **2004**, *74*, 83-92.
- [74] Aartsma-Rus, A.; Janson, A. A.; Kaman, W. E.; Bremmer-Bout, M.; den Dunnen, J. T.; Baas, F.; van Ommen, G. J.; van Deutekom, J. C. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Hum. Mol. Genet.* **2003**, *12*, 907-914.
- [75] Aartsma-Rus, A.; Bremmer-Bout, M.; Janson, A. A.; den Dunnen, J. T.; van Ommen, G. J.; van Deutekom, J. C. Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. *Neuromuscul. Disord.* **2002**, *12*(Suppl. 1), S71-S77.
- [76] Beroud, C.; Tuffery-Giraud, S.; Matsuo, M.; Hamroun, D.; Humbertclaude, V.; Monnier, N.; Moizard, M. P.; Voelckel, M. A.; Caemard, L. M.; Boisseau, P.; Blayau, M.; Philippe, C.; Cossee, M.; Pages, M.; Rivier, F.; Danos, O.; Garcia, L.; Claustres, M. Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. *Hum. Mutat.* **2007**, *28*, 196-202.
- [77] Aartsma-Rus, A.; De Winter, C. L.; Janson, A. A.; Kaman, W. E.; Van Ommen, G. J.; Den Dunnen, J. T.; Van Deutekom, J. C. Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites. *Oligonucleotides* **2005**, *15*, 284-297.
- [78] Wilton, S. D.; Fall, A. M.; Harding, P. L.; McClorey, G.; Coleman, C.; Fletcher, S. Antisense Oligonucleotide-induced Exon Skipping Across the Human Dystrophin Gene Transcript. *Mol. Ther.* **2007**, *15*, 1288-1296.
- [79] Aartsma-Rus, A.; van Ommen, G. J. Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications. *RNA* **2007**, *13*(10), 1609-1624.
- [80] Cartegni, L.; Wang, J.; Zhu, Z.; Zhang, M. Q.; Krainer, A. R. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res.* **2003**, *31*, 3568-3571.
- [81] Smith, P. J.; Zhang, C.; Wang, J.; Chew, S. L.; Zhang, M. Q.; Krainer, A. R. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. *Hum. Mol. Genet.* **2006**, *15*, 2490-2508.
- [82] Wang, G. S.; Cooper, T. A. Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat. Rev. Genet.* **2007**, *8*, 749-761.
- [83] Aartsma-Rus, A.; Kaman, W. E.; Weij, R.; den Dunnen, J. T.; van Ommen, G. J.; van Deutekom, J. C. Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. *Mol. Ther.* **2006**, *14*, 401-407.
- [84] Cartegni, L.; Chew, S. L.; Krainer, A. R. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat. Rev. Genet.* **2002**, *3*, 285-298.
- [85] Errington, S. J.; Mann, C. J.; Fletcher, S.; Wilton, S. D. Target selection for antisense oligonucleotide induced exon skipping in the dystrophin gene. *J. Gene Med.* **2003**, *5*, 518-527.
- [86] Harding, P. L.; Fall, A. M.; Honeyman, K.; Fletcher, S.; Wilton, S. D. The influence of antisense oligonucleotide length on dystrophin exon skipping. *Mol. Ther.* **2007**, *15*, 157-166.
- [87] Adams, A. M.; Harding, P. L.; Iversen, P. L.; Coleman, C.; Fletcher, S.; Wilton, S. D. Antisense oligonucleotide induced exon skipping and the dystrophin gene transcript: cocktails and chemistries. *BMC Mol. Biol.* **2007**, *8*, 57.
- [88] Makeyev, E. V.; Maniatis, T. Multilevel regulation of gene expression by microRNAs. *Science* **2008**, *319*, 1789-1790.
- [89] Arechavala-Gomez, V.; Graham, I. R.; Popplewell, L. J.; Adams, A. M.; Aartsma-Rus, A.; Kinali, M.; Morgan, J. E.; Van Deutekom, J. C.; Wilton, S. D.; Dickson, G.; Muntoni, F. Comparative Analysis of Antisense Oligonucleotide Sequences for Targeted Skipping of Exon 51 During Dystrophin Pre-mRNA Splicing in Human Muscle. *Hum. Gene Ther.* **2007**, *18*, 798-810.
- [90] Buratti, E.; Baralle, F. E. Influence of RNA secondary structure on the pre-mRNA splicing process. *Mol. Cell Biol.* **2004**, *24*, 10505-10514.
- [91] Buratti, E.; Muro, A. F.; Giombi, M.; Gherbassi, D.; Iaconcig, A.; Baralle, F. E. RNA folding affects the recruitment of SR proteins by mouse and human polypurinic enhancer elements in the fibronectin EDA exon. *Mol. Cell Biol.* **2004**, *24*, 1387-1400.
- [92] Hiller, M.; Zhang, Z.; Backofen, R.; Stamm, S. Pre-mRNA Secondary Structures Influence Exon Recognition. *PLoS Genet.* **2007**, *3*, e204.
- [93] Takeshima, Y.; Wada, H.; Yagi, M.; Ishikawa, Y.; Ishikawa, Y.; Minami, R.; Nakamura, H.; Matsuo, M. Oligonucleotides against a splicing enhancer sequence led to dystrophin production in muscle cells from a Duchenne muscular dystrophy patient. *Brain Dev.* **2001**, *23*, 788-790.
- [94] Takeshima, Y.; Yagi, M.; Wada, H.; Ishibashi, K.; Nishiyama, A.; Kakumoto, M.; Sakaeda, T.; Saura, R.; Okumura, K.; Matsuo, M. Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. *Pediatr. Res.* **2006**, *59*, 690-694.
- [95] Yagi, M.; Takeshima, Y.; Surono, A.; Takagi, M.; Koizumi, M.; Matsuo, M. Chimeric RNA and 2'-O, 4'-C-ethylene-bridged nucleic acids have stronger activity than phosphorothioate oligodeoxynucleotides in induction of exon 19 skipping in dystrophin mRNA. *Oligonucleotides* **2004**, *14*, 33-40.
- [96] Mann, C. J.; Honeyman, K.; Cheng, A. J.; Ly, T.; Lloyd, F.; Fletcher, S.; Morgan, J. E.; Partridge, T. A.; Wilton, S. D. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 42-47.
- [97] Gebiski, B. L.; Errington, S. J.; Johnsen, R. D.; Fletcher, S.; Wilton, S. D. Terminal antisense oligonucleotide modifications can enhance induced exon skipping. *Neuromuscul. Disord.* **2005**, *15*, 622-629.
- [98] Surono, A.; Van Khanh, T.; Takeshima, Y.; Wada, H.; Yagi, M.; Takagi, M.; Koizumi, M.; Matsuo, M. Chimeric RNA/ethylene-bridged nucleic acids promote dystrophin expression in myocytes of Duchenne muscular dystrophy by inducing skipping of the nonsense mutation-encoding exon. *Hum. Gene Ther.* **2004**, *15*, 749-757.
- [99] Ittig, D.; Liu, S.; Renneberg, D.; Schumperli, D.; Leumann, C. J. Nuclear antisense effects in cyclophilin A pre-mRNA splicing by oligonucleotides: a comparison of tricyclo-DNA with LNA. *Nucleic Acids Res.* **2004**, *32*, 346-353.
- [100] Gebiski, B. L.; Mann, C. J.; Fletcher, S.; Wilton, S. D. Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. *Hum. Mol. Genet.* **2003**, *12*, 1801-1811.
- [101] Muntoni, F.; Bushby, K.; van Ommen, G. 128th ENMC International Workshop on 'Preclinical optimization and Phase I/II Clinical Trials Using Antisense Oligonucleotides in Duchenne Muscular Dystrophy' 22-24 October 2004, Naarden, The Netherlands. *Neuromuscul. Disord.* **2005**, *15*, 450-457.
- [102] van Deutekom, J. C.; Janson, A. A.; Ginjaar, I. B.; Frankhuizen, W. S.; Aartsma-Rus, A.; Bremmer-Bout, M.; den Dunnen, J. T.; Koop, K.; van der Kooi, A. J.; Goemans, N. M.; de Kimpe, S. J.; Ekhart, P. F.; Venneker, E. H.; Platenburg, G. J.; Verschuuren, J. J.; van Ommen, G. J. Local dystrophin restoration with antisense oligonucleotide PRO051. *N. Engl. J. Med.* **2007**, *357*, 2677-2686.
- [103] Yin, H.; Lu, Q.; Wood, M. Effective Exon Skipping and Restoration of Dystrophin Expression by Peptide Nucleic Acid Antisense Oligonucleotides in mdx Mice. *Mol. Ther.* **2008**, *16*, 38-45.
- [104] Schmajuk, G.; Sierakowska, H.; Kole, R. Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. *J. Biol. Chem.* **1999**, *274*, 21783-21789.
- [105] Iversen, P. L. In: *Morpholino, in Antisense drug technology: Principles, Strategies, and Applications*; S. T. Crooke, Ed.; Taylor and Francis Group: **2007**, pp. 556-582.
- [106] Lu, Q. L.; Mann, C. J.; Lou, F.; Bou-Gharios, G.; Morris, G. E.; Xue, S. A.; Fletcher, S.; Partridge, T. A.; Wilton, S. D. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat. Med.* **2003**, *9*, 1009-1014.
- [107] Lu, Q. L.; Rabinowitz, A.; Chen, Y. C.; Yokota, T.; Yin, H.; Alter, J.; Jadoon, A.; Bou-Gharios, G.; Partridge, T. Systemic delivery of antisense oligonucleotide restores dystrophin expression in body-wide skeletal muscles. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 198-203.
- [108] Wells, K. E.; Fletcher, S.; Mann, C. J.; Wilton, S. D.; Wells, D. J. Enhanced *in vivo* delivery of antisense oligonucleotides to restore dystrophin expression in adult mdx mouse muscle. *FEBS Lett.* **2003**, *552*, 145-149.
- [109] Fletcher, S.; Honeyman, K.; Fall, A. M.; Harding, P. L.; Johnsen, R. D.; Wilton, S. D. Dystrophin expression in the mdx mouse after localised and systemic administration of a morpholino antisense oligonucleotide. *J. Gene Med.* **2006**, *8*, 207-216.
- [110] Alter, J.; Lou, F.; Rabinowitz, A.; Yin, H.; Rosenfeld, J.; Wilton, S. D.; Partridge, T. A.; Lu, Q. L. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat. Med.* **2006**, *12*, 175-177.
- [111] Fletcher, S.; Honeyman, K.; Fall, A. M.; Harding, P. L.; Johnsen, R. D.; Steinhaus, J. P.; Moulton, H. M.; Iversen, P. L.; Wilton, S. D. Morpholino Oligomer-Mediated Exon Skipping Averts the Onset of Dystrophic Pathology in the mdx Mouse. *Mol. Ther.* **2007**, *15*, 1587-1592.
- [112] Bertoni, C.; Rando, T. A. Dystrophin gene repair in mdx muscle precursor cells *in vitro* and *in vivo* mediated by RNA-DNA chimeric oligonucleotides. *Hum. Gene Ther.* **2002**, *13*, 707-718.
- [113] Bertoni, C.; Lau, C.; Rando, T. A. Restoration of dystrophin expression in mdx muscle cells by chimeraplast-mediated exon skipping. *Hum. Mol. Genet.* **2003**, *12*, 1087-1099.
- [114] Bertoni, C.; Morris, G. E.; Rando, T. A. Strand bias in oligonucleotide-mediated dystrophin gene editing. *Hum. Mol. Genet.* **2005**, *14*, 221-233.
- [115] Bertoni, C.; Jarrahan, S.; Wheeler, T. M.; Li, Y.; Olivares, E. C.; Calos, M. P.; Rando, T. A. Enhancement of plasmid-mediated gene therapy for muscular dystrophy by directed plasmid integration. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 419-424.
- [116] Wilton, S. D.; Chandler, D. C.; Kakulas, B. A.; Laing, N. G. Identification of a point mutation and germinal mosaicism in a Duchenne muscular dystrophy family. *Hum. Mutat.* **1994**, *3*, 133-140.
- [117] Taubes, G. Gene therapy. The strange case of chimeraplasty. *Science* **2002**, *298*, 2116-2120.
- [118] De Meyer, S. F.; Pareyn, I.; Baert, J.; Deckmyn, H.; Vanhoorelbeke, K. False positive results in chimeraplasty for von Willebrand Disease. *Thromb. Res.* **2007**, *119*, 93-104.
- [119] Dentí, M. A.; Rosa, A.; D'Antona, G.; Standler, O.; De Angelis, F. G.; Nicoletti, C.; Allocca, M.; Pansarasa, O.; Parente, V.; Musaro, A.; Auricchio,

- A.; Bottinelli, R.; Bozzoni, I. Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice. *Hum. Gene Ther.* **2006**, *17*, 565-574.
- [120] Denti, M. A.; Rosa, A.; D'Antona, G.; Sthandier, O.; De Angelis, F. G.; Nicoletti, C.; Allocca, M.; Pansarasa, O.; Parente, V.; Musaro, A.; Auricchio, A.; Bottinelli, R.; Bozzoni, I. Body-wide gene therapy of Duchenne muscular dystrophy in the mdx mouse model. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3758-3763.
- [121] Goyenvalle, A.; Vulin, A.; Fougereuse, F.; Leturcq, F.; Kaplan, J. C.; Garcia, L.; Danos, O. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* **2004**, *306*, 1796-1799.
- [122] Torrente, Y.; Belicchi, M.; Marchesi, C.; Dantona, G.; Cogiamanian, F.; Pisati, F.; Gavina, M.; Giordano, R.; Tonlorenzi, R.; Fagiolari, G.; Lamperti, C.; Porretti, L.; Lopa, R.; Sampaolesi, M.; Vicentini, L.; Grimoldi, N.; Tiberio, F.; Songa, V.; Baratta, P.; Prele, A.; Forzenigo, L.; Guglieri, M.; Pansarasa, O.; Rinaldi, C.; Mouly, V.; Butler-Browne, G. S.; Comi, G. P.; Biondetti, P.; Moggio, M.; Gai, S. M.; Stocchetti, N.; Priori, A.; D'Angelo, M. G.; Turconi, A.; Bottinelli, R.; Cossu, G.; Rebull, P.; Bresolin, N. Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients. *Cell Transplant.* **2007**, *16*, 563-577.
- [123] Quenneville, S. P.; Chapdelaine, P.; Skuk, D.; Paradis, M.; Goulet, M.; Rousseau, J.; Xiao, X.; Garcia, L.; Tremblay, J. P. Autologous transplantation of muscle precursor cells modified with a lentivirus for muscular dystrophy: human cells and primate models. *Mol. Ther.* **2007**, *15*, 431-438.
- [124] Benchaouir, R.; Merigalli, M.; Farini, A.; D'Antona, G.; Belicchi, M.; Goyenvalle, A.; Battistelli, M.; Bresolin, N.; Bottinelli, R.; Garcia, L.; Torrente, Y. Restoration of Human Dystrophin Following Transplantation of Exon-Skipping-Engineered DMD Patient Stem Cells into Dystrophic Mice. *Cell Stem Cell* **2007**, *1*, 646-657.
- [125] Takeshima, Y.; Yagi, M.; Wada, H.; Matsuo, M. Intraperitoneal administration of phosphorothioate antisense oligodeoxynucleotide against splicing enhancer sequence induced exon skipping in dystrophin mRNA expressed in mdx skeletal muscle. *Brain Dev.* **2005**, *27*, 488-493.
- [126] Roberts, R. G.; Bentley, D. R.; Bobrow, M. Infidelity in the structure of ectopic transcripts: a novel exon in lymphocyte dystrophin transcripts. *Hum. Mutat.* **1993**, *2*, 293-299.
- [127] Chelly, J.; Kaplan, J. C.; Maire, P.; Gautron, S.; Kahn, A. Transcription of the dystrophin gene in human muscle and non-muscle tissue. *Nature* **1988**, *333*, 858-860.
- [128] Fanin, M.; Freda, M. P.; Vitiello, L.; Danieli, G. A.; Pegoraro, E.; Angelini, C. Duchenne phenotype with in-frame deletion removing major portion of dystrophin rod: threshold effect for deletion size? *Muscle Nerve* **1996**, *19*, 1154-1160.
- [129] Adams, C. P.; Brantner, V. V. Estimating the cost of new drug development: is it really 802 million dollars? *Health Aff (Millwood)* **2006**, *25*, 420-428.
- [130] Madden, H.; Fletcher, S.; Davis, M.; Wilton, S. D. Characterisation of a complex DMD-causing dystrophin gene inversion and restoration of the reading frame by induced exon skipping. *Hum. Mutat.* **2008**, [Epub ahead of print].
- [131] Sambrook, P.; Lane, N. E. Corticosteroid osteoporosis. *Best Pract. Res. Clin. Rheumatol.* **2001**, *15*, 401-413.
- [132] Yu, S.; Holsboer, F.; Almeida, O. F. Neuronal actions of glucocorticoids: Focus on depression. *J. Steroid Biochem. Mol. Biol.* **2008**, *108*, 300-309.
- [133] Manzur, A. Y.; Kuntzer, T.; Pike, M.; Swan, A. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. *Cochrane Database Syst. Rev.* **2004**, *CD003725*, 1-65.
- [134] Gospe, S. M., Jr.; Lazaro, R. P.; Lava, N. S.; Grootsholten, P. M.; Scott, M. O.; Fischbeck, K. H. Familial X-linked myalgia and cramps: a nonprogressive myopathy associated with a deletion in the dystrophin gene. *Neurology* **1989**, *39*, 1277-1280.
- [135] Ishigaki, C.; Patria, S. Y.; Nishio, H.; Yabe, M.; Matsuo, M. A Japanese boy with myalgia and cramps has a novel in-frame deletion of the dystrophin gene. *Neurology* **1996**, *46*, 1347-1350.
- [136] Morandi, L.; Mora, M.; Confalonieri, V.; Barresi, R.; Di Blasi, C.; Brugnoli, R.; Bernasconi, P.; Mantegazza, R.; Dworzak, F.; Antozzi, C.; Balestrini, M. R.; Jarre, L.; Pini, A.; Merlini, L.; Piccolo, G.; Mazzanti, A.; Daniel, S.; Bläsewich, F.; Cornelio, F. Dystrophin characterization in BMD patients: correlation of abnormal protein with clinical phenotype. *J. Neurol. Sci.* **1995**, *132*, 146-155.
- [137] Mirabella, M.; Galluzzi, G.; Manfredi, G.; Bertini, E.; Ricci, E.; De Leo, R.; Tonali, P.; Servidei, S. Giant dystrophin deletion associated with congenital cataract and mild muscular dystrophy. *Neurology* **1998**, *51*, 592-595.
- [138] Koenig, M.; Beggs, A. H.; Moyer, M.; Scherpf, S.; Heindrich, K.; Bettecken, T.; Meng, G.; Muller, C. R.; Lindlof, M.; Kaariainen, H.; de la Chapelle, A.; Kiuru, A.; Savontaus, M.-L.; Gilgenkrantz, H.; Récan, D.; Chelly, J.; Kaplan, J.-C.; Covone, A. E.; Archidiacono, N.; Romeo, G.; Liechti-Gallati, S.; Schneider, V.; Braga, S.; Moser, H.; Darras, B. T.; Murphy, P.; Francke, U.; Chen, J. D.; Morgan, G.; Denton, M.; Greenberg, C. R.; Wrogemann, K.; Blonden, L. A. J.; van Paassen, H. M. B.; van Ommen, G. J. B.; Kunkel, L. M. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am. J. Hum. Genet.* **1989**, *45*, 498-506.
- [139] Comi, G. P.; Prele, A.; Bresolin, N.; Moggio, M.; Bardoni, A.; Gallanti, A.; Vita, G.; Toscano, A.; Ferro, M. T.; Bordoni, A.; Fortunato, F.; Ciscato, P.; Felisari, G.; Tedeschi, S.; Castelli, E.; Garghentino, R.; Turconi, A.; Fraschini, P.; Marchi, E.; Negretto, G. G.; Adobbati, L.; Meola, G.; Tonin, P.; Papadimitriou, A.; Scarlato, G. Clinical variability in Becker muscular dystrophy. Genetic, biochemical and immunohistochemical correlates. *Brain* **1994**, *117 (Pt 1)*, 1-14.
- [140] Bosone, I.; Bortolotto, S.; Mongini, T.; Doriguzzi, C.; Chiado-Piat, L.; Ugo, I.; Mutani, R.; Palmucci, L. Late onset and very mild course of Xp21 Becker type muscular dystrophy. *Clin. Neuropathol.* **2001**, *20*, 196-199.
- [141] Morrone, A.; Zammarchi, E.; Scacheri, P. C.; Donati, M. A.; Hoop, R. C.; Servidei, S.; Galluzzi, G.; Hoffman, E. P. Asymptomatic dystrophinopathy. *Am. J. Med. Genet.* **1997**, *69*, 261-267.
- [142] Beggs, A. H.; Hoffman, E. P.; Snyder, J. R.; Arahata, K.; Specht, L.; Shapiro, F.; Angelini, C.; Sugita, H.; Kunkel, L. M. Exploring the molecular basis for variability among patients with Becker muscular dystrophy: dystrophin gene and protein studies. *Am. J. Hum. Genet.* **1991**, *49*, 54-67.