Abstract

Exon skipping using antisense oligonucleotides (AONs) has successfully been used to reframe the mRNA in various DMD (Duchenne muscular dystrophy) patients carrying deletions and in the *mdx* mouse model. This study can be devided in two parts: in the first part we have tested the feasibility of the exon skipping approach for patients with small mutations in in-frame exons, while in the second part a quantitative comparison of exon skipping revealing techniques is addressed.

We first identified 55 novel disease-causing point mutations. We selected 5 patients with nonsense or frameshifting mutations in exons 10, 16, 26, 33 and 34. Wild type and mutation specific 2'OMePS AONs were tested in cell-free splicing assays and in cultured cells derived from the selected patients. The results obtained confirm cell-free splicing assay as an alternative system to test exon skipping propensity when patients' cells are unavailable. In myogenic cells, similar levels of exon skipping were observed for wild type and mutation specific AONs for exons 16, 26 and 33, while for exon 10 and exon 34 the efficiency of the AONs was significantly different. Interestingly, in some cases skipping efficiencies for mutated exons were quite dissimilar compared to what previously reported for the respective wild type exons. This behaviour may be related to effect of the mutations on exon skipping propensity and highlights the complexity of identifying optimal AONs for skipping exons with small mutations.

In the second part we compared different techniques to reveal the exon skipping levels in the muscles of 7 different *mdx* mice. An absolute quantification of the dystrophin transcript amount was possible using a digital array. Results underline the low expression of the dytrophin gene and the amount needed to correctly quantify the exon skipping percentage.

List of Publications

I **Spitali P**, Rimessi P, Fabris M, Perrone D, Falzarano S, Bovolenta M, Trabanelli C, Mari L, Bassi E, Tuffery S, Gualandi F, Maraldi NM, Sabatelli-Giraud P, Medici A, Merlini L, Ferlini A. Exon skipping-mediated dystrophin reading frame restoration for small mutations. Hum Mutat. 2009 30:1527-34.

II Rimessi P, Sabatelli P, Fabris M, Braghetta P, Bassi E, **Spitali P**, Vattemi G, Tomelleri G, Mari L, Perrone D, Medici A, Neri M, Bovolenta M, Martoni E, Maraldi NM, Gualandi F, Merlini L, Ballestri M, Tondelli L, Sparnacci K, Bonaldo P, Caputo A, Laus M, Ferlini A. Cationic PMMA nanoparticles bind and deliver antisense oligoribonucleotides allowing restoration of dystrophin expression in the mdx mouse. Mol Ther. 2009 7:820-7.

III **Spitali P**, Heemskerk JA, Vossen R.H.A.M, Ferlini A, den Dunnen J.T., 't Hoen P.A.C. and Aartsma-Rus A. Accurate quantification of dystrophin mRNA and exon skipping levels in Duchenne Muscular Dystrophy. Laboratory Investigation. Manuscipt in Press.

Contents

List of abbreviations

2OMe	2'-O-methyl modified
AAV	Adeno-associated virus
ABD	Actin binding domain
AON	Antisense oligonucleotide
BMD	Becker muscular dystrophy
DGC	Dystrophin associated glycoprotein complex
DGGE	Denaturant gradient gel electrophoresis\
DMD	Duchenne muscular dystrophy
DNA	Deoxiribose nucleic acid
DRP2	Dystrophin related protein 2
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
GRMD	Golden retriever muscular dystrophy
HFMD	Hypertrophic feline muscular dystrophy
hnRNP	Heterogeneous nuclear ribonucleoprotein
LGMD	Limb-girdle muscular dystrophy
LNA	Locked nucleic acid
nNOS	Neuronal nitric oxide synthase
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PEI	Polyethylenimine
PMMA	polymethylmethacrylate
PMO	morpholino oligonucleotides
PNA	Peptide nucleic acid
PPMO	peptide-conjugated morpholino oligonucleoides
PPNA	peptide-conjugated peptide nucleic acid
PS	Phosphorothioate
PTT	Protein truncation test
RNA	Ribose nucleic acid
RT-PCR	Reverse transcription polymerase chain reaction

snRNA	Small nuclear ribonucleoprotein
SCAIP	single-condition amplification/internal primer
SSCA	Single strand conformation polymorphism analysis
U2AF	U2 auxillary factor
UTR	Untranslated region
XLDC	X-linked dilated cardiomyopathy

1. Phenotype

Duchenne muscular dystrophy (DMD) is a severe, progressive, muscle-wasting neuromuscular disorder (Emery 2002). The worldwide incidence of DMD is estimated to be 1 in 3500 newborn boys (Moser 1984). The first symptoms involve the lower limbs and appear between the third and fifth year. Patients have hypertrophic calves, show difficulty in running and climbing stairs, run on their tiptoes and frequently fall. Due to weakness of the knee and hip extensors, patients rise from a sitting position using the Gower's maneuver (patients rest on their hands and toes, and then use their hands to push down on their thighs to get into an upright position). Muscle weakness progresses to the shoulder girdle-, upper armand trunk-muscles and patients loose ambulance before the age of 12 (a detailed description of the clinical features can be found in (Emery 1993)).

Histological changes are readily apparent with light microscopy analysis of cross-sections from patient muscle biopsies (Figure 1). They involve variation in fiber size with atrophic and hypertrophic fibers, degeneration and regeneration of the muscle fibers, infiltration of inflammatory cells and fibrosis (Dubowitz 2000). The fiber necrosis results in leakage of the enzyme creatine kinase (CK), resulting in very high serum CK levels in DMD patients (20,000 to 50,000 U/L compared to 80 to 250 U/L in unaffected individuals). These levels decline as patients get older and the overall muscle mass decreases progressively.

Until the early nineties of the last century, patients used to die from respiratory insufficiency in the second decade of their life. Nowadays, patients live into their third decade, due to improved respiratory care and assisted ventilation, and often die as a result of congestive cardio-myopathy (Emery 2002; Simonds 1998). A substantial proportion, however, still dies before their twenties from pneumonia or other respiratory infections.

One third of all affected boys are also mentally impaired. About 20% of DMD patients have an IQ of less than 70 (Emery 2002) and learning difficulties are commonly associated with the disease (Polakoff 1998). In contrast to the muscle wasting, the mental impairment is not progressive.
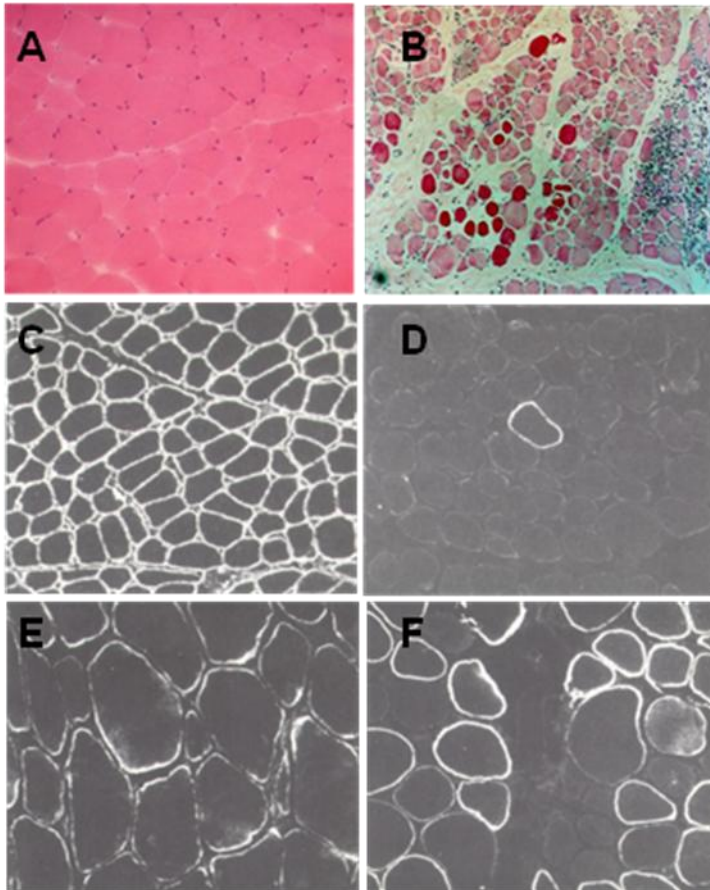
Figure 1. Hematoxilin and eosin staining (A-B) and dystrophin immuno-histochemical staining (using dys2) (C-F) of muscle cross sections. **A**. Healthy human skeletal muscle consists of fibers that are evenly spaced. The fibers are multinucleated with nuclei located at the periphery of the fibers. **B**. DMD muscle is characterized by necrotic fibers degeneration) and infiltration of inflammatory cells. Degenerating fibers with nuclei located in the center of the fibers are also found. **C**. Dystrophin is present at the sarcolemma in normal human muscle. **D**. Dystrophin is absent in muscle fibers from DMD patients. **E**. In most BMD patients dystrophin shows a less intense, discontinuous staining. **F**. In carriers random X-inactivation results in both dystrophin positive and dystrophin negative fibers. (Pictures kindly provided by dr. Alesandra Ferlini).
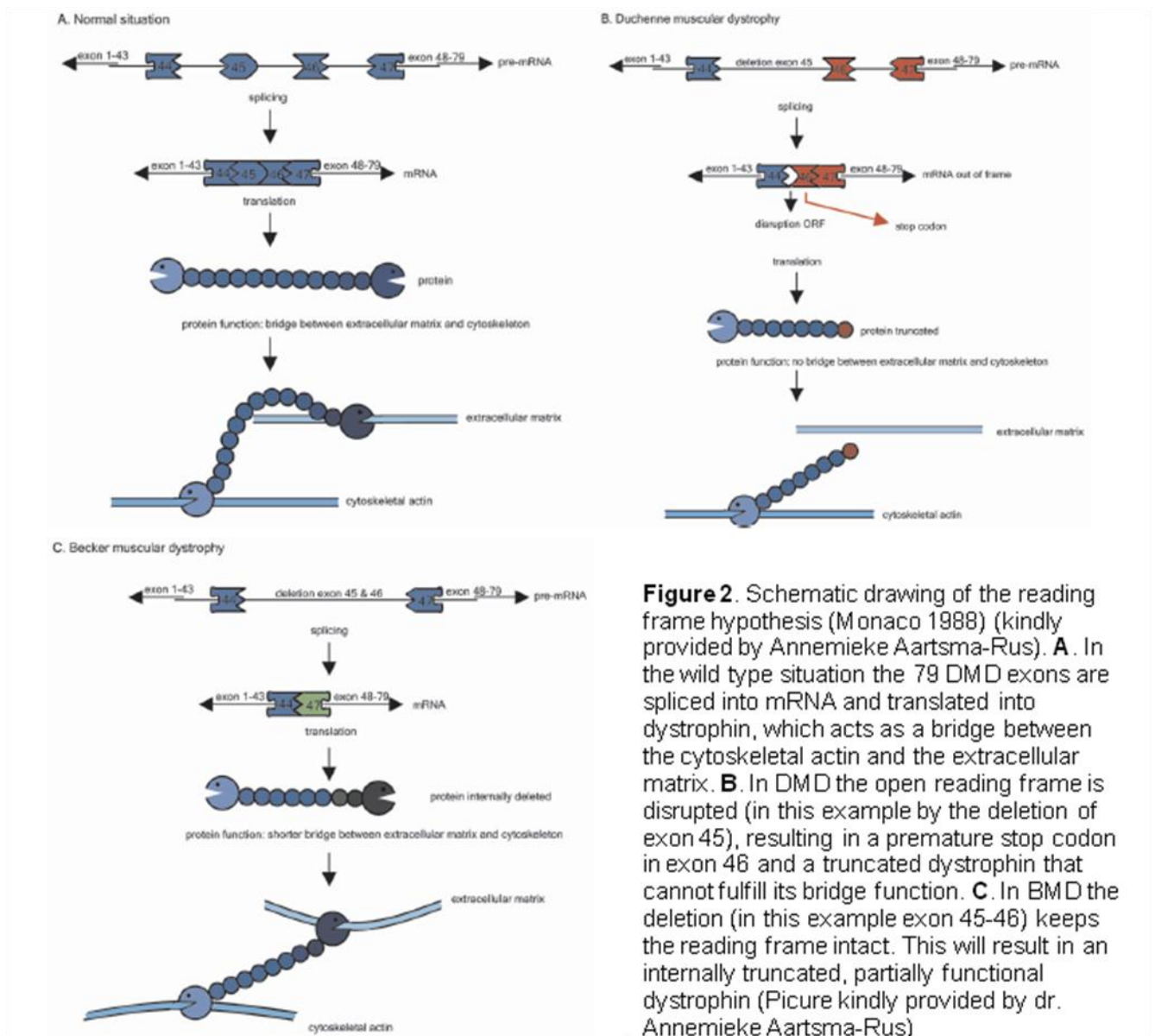
2. Positionl cloning of the DMD gene

Since in general only boys are affected by DMD, it was obvious to geneticists that the gene mutated in DMD patients, had to be located on the X-chromosome. A few, rare, cases of female DMD patients have been described. In the majority of these females, the disease was associated with translocations in the Xp21 band, indicating that the DMD locus was located in this region (Greenstein 1977; Jacobs 1981). Besides this cytogenetical evidence, genetic linkage analysis also localized the DMD locus to the Xp21 band (Davies 1983). Using probes located in and around this band prenatal diagnosis and carrier detection was performed as early as 1985 (Bakker 1985).

Surprisingly, linkage analysis localized Becker muscular dystrophy (BMD) to the same locus as DMD (Kingston 1984). BMD affects 1 in 20,000 men, and patients also suffer from muscle weakness. However, the course of the disease is more benign than for DMD (Emery 2002). Patients have a phenotype that varies from very mild to moderately severe. The age of onset is around 12 years, but some patients remain asymptomatic until later in life. Most patients lose ambulance around 20-30 years after diagnosis (Emery 1993), although some remain ambulant for much longer (England 1990;

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Mirabella 1998; Yazaki 1999). About 50% of all BMD patients suffer from cardiomyopathy, which sometimes is the main symptom (de Visser 1990; Emery 1993). Severely affected patients die between 40 and 50 years, whereas mildly affected patients have near normal life expectancies. The pERT87 probe, which is deleted in some DMD patients, was shown to be deleted in several BMD patients as well (Hart 1987), suggesting that DMD and BMD are either allelic or that the genes mutated in these diseases are close together. Using different probes the first partial DMD cDNAs were identified by Monaco and collegues in 1986 (Monaco 1986), whereupon the complete 14 kb DMD cDNA could be cloned in 1987 (Koenig 1987). The gene (described in more detail in Chapter 3) was found to contain at least 60 exons and to be dispersed over at least 2000 kb. Deletions in the DMD gene were found in 50% of all patients using cDNA probes (den Dunnen 1987; Koenig 1987). Following studies found similar or even higher percentages of deletions in DMD patients as well as in BMD patients (Davies 1988; Den Dunnen 1989; Forrest 1988). The protein product of the DMD gene was identified shortly after the DMD cDNA. It was named 'dystrophin', because of its identification via the isolation of the Duchenne muscular dystrophy locus (Hoffman 1987). The protein was shown to be approximately 400 kD and to represent 0.002% of total striated muscle protein (Hoffman 1987). Dystrophin could not be detected in muscle tissue isolated from DMD patients. Microscopic analysis of cross-sections revealed that dystrophin is located in the sarcolemma in unaffected muscle tissue, and confirmed that it is absent in muscles from DMD patients (Arahata 1988; Bonilla 1988; Watkins 1988; Zubrzycka-Gaarn 1988) (Figure 1C-F). In contrast to DMD patients, dystrophin could be detected in BMD patients (Figure 1E). Western blot analysis revealed that these dystrophins were of an abnormal size, but present in near normal levels in mildly affected BMD patients, and in low amounts for more severely affected patients (Hoffman 1988). These findings implied that the amount of the protein was more important than the size of the protein. In 1988 Monaco and colleagues proposed the open reading frame hypothesis, by which the phenotypic differences between DMD and BMD patients could be explained (Monaco 1988). This model was based on the breakpoints of intragenic DMD deletions and their effect on the translation of triplet codons into amino acids of the dystrophin protein. They hypothesized that deletions in DMD patients lead to a shift in the open reading frame, resulting in premature stop codons and truncated non-functional, instable proteins, associated with

the severe DMD phenoptype. In BMD patients the deletions do not disrupt the open reading frame and internally deleted, but semi-functional dystrophins can be produced, leading to a milder phenotype (See Figure 2 for a schematic depiction). Some studies detected mutations that contradicted this 'open reading frame rule', and suggested that the location of the mutation determined the outcome of the disease (Malhotra 1988; Medori 1989). However, the vast majority of these exceptions consisted of BMD patients carrying an out-of-frame deletion of exon 3-7 (discussed in more detail in Chapter 6). An extensive study revealed that for mutations detected on DNA level the reading frame rule holds true for over 90% of all BMD and DMD patients (Baumbach 1989; Gillard 1989; Koenig 1989), and this rule is generally used as a differential diagnostic and prognostic tool for BMD versus DMD patients.

Figure 2. Schematic drawing of the reading frame hypothesis (Monaco 1988) (kindly provided by Annemieke Aartsma-Rus). **A**. In the wild type situation the 79 DMD exons are spliced into mRNA and translated into dystrophin, which acts as a bridge between the cytoskeletal actin and the extracellular matrix. **B**. In DMD the open reading frame is disrupted (in this example by the deletion of exon 45), resulting in a premature stop codon in exon 46 and a truncated dystrophin that cannot fulfill its bridge function. **C**. In BMD the deletion (in this example exon 45-46) keeps the reading frame intact. This will result in an internally truncated, partially functional dystrophin (Picure kindly provided by dr. Annemieke Aartsma-Rus)

3. The DMD gene

The DMD gene is the largest known gene and consists of no less than 2,2 Mb, making it 80 times as big as the average human gene (estimated to be 30 kb (Nebert 2002)). The gene contributes to approximately 0.1 % of the human genome (Lander 2001). When compared to the average gene (spanning ~6 kb and containing ~10 exons (Lewin 2000)), the DMD mRNA is larger (14 kb) and contains more exons (79) (Roberts 1993). However, there are genes that contain even more exons and have larger mRNAs than the DMD gene, but which are nevertheless shorter. This can be explained by the fact that the coding sequence of the DMD gene accounts for only 0.6% of the gene and the rest of the gene is occupied by huge non-coding introns (Ahn 1993) (Figure 3). The dystrophin gene is also unusually large in other organisms, such as the mouse (Mus musculus; 2.2 Mb where the average murine gene is 27 kb and the pufferfish (Fugu rubripes; 165kb vs. 4,8 kb for an average gene); and much larger than the average gene in the fruitfly (Drosophila melanogaster; 131 kb vs. 11,3 kb for an average gene).
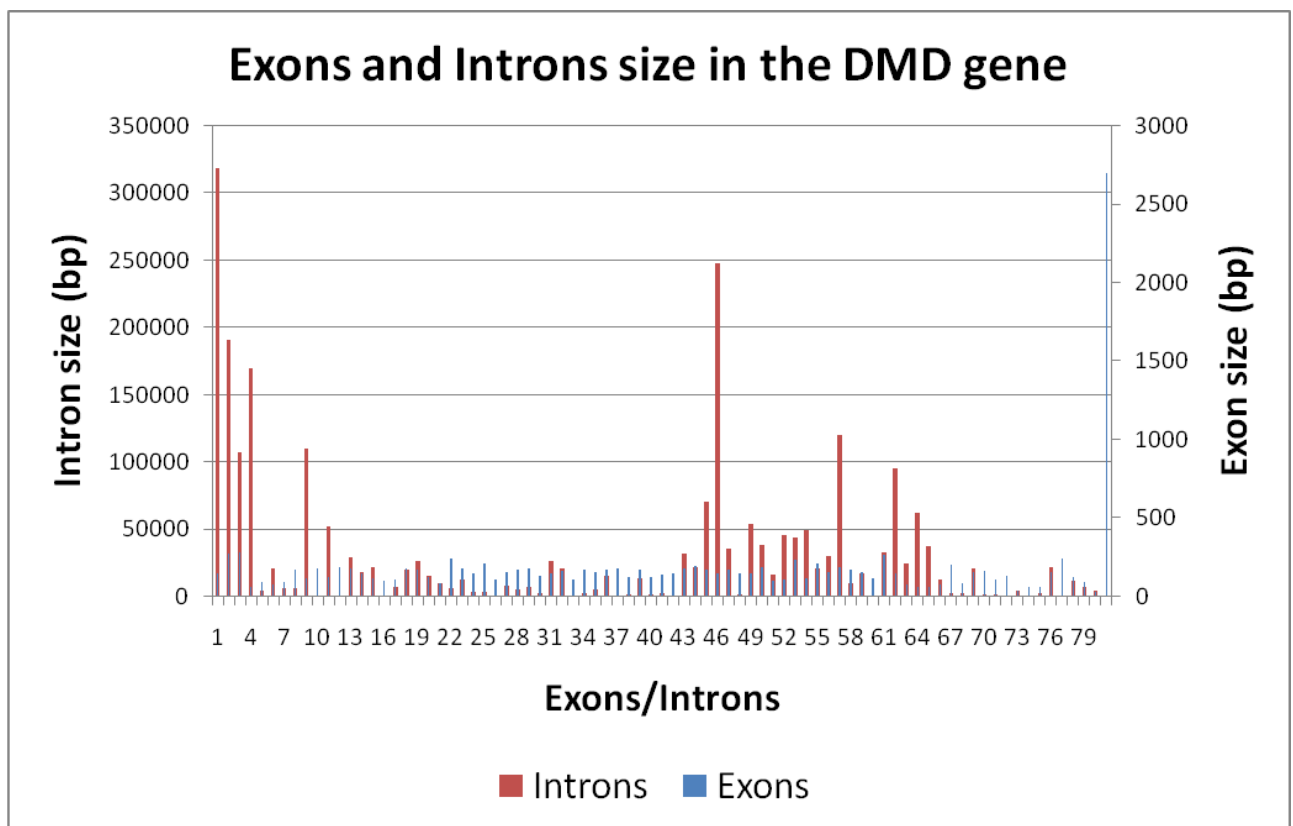

Figure 3. Exons and introns size in the DMD gene. Exons (blue bars) an introns (red bars) are indicated.

Since the extreme size of the gene makes it very vulnerable for pathological rearrangements, the question arises why this gene is so large. One hypothesis is, that it prevents the dystrophin protein from being present too early during myogenesis and brain formation. Full-length dystrophins are mainly transcribed in post-mitotic cells in muscle and brain. During mitosis, there is a decrease in transcription and it would be almost impossible to express a gene that takes an estimated 16 hours to transcribe (Tennyson 1995) in early embryonic cells that divide every 24 hours. Another hypothesis is that the introns contain regulatory elements for (alternative) splicing events. This theory is supported by the fact that in-frame exons, which can be skipped without serious consequences, are on average flanked by smaller introns than out-of-frame exons, which will result in the generation of a truncated, non- functional dystrophin when abusively skipped (Pozzoli 2003).

The huge size of the introns requires an enormous effort of the transcription and splicing machinery of the cell. Furthermore, the predicted splice sites of the DMD exons are not significantly stronger than those of other genes (Sironi 2001), yet the splicing machinery manages to correctly splice the 14 kb mRNA from the 2.4 Mb pre-mRNA for the majority of the transcripts. This is even more astonishing if you appreciate that for instance intron 44 is almost 250 kb long, and that exon 2 is flanked by 360 kb of intron sequence (i.e. 190 kb for intron 1 and 170 kb for intron 2), in which many other potential exons are located, with equally good or better splice sites than the exon itself. It may be that the genuine exons are recognized due to additional signals present in the introns, or due to the secondary structure of the pre-mRNA.

The dystrophin pre-mRNA is subjected to alternative splicing throughout its coding sequence (Sironi 2002). In the C-terminal part the in-frame exon 71 and the out-of-frame exon 78 are omitted in 50% of dystrophin transcripts (Feener 1989). The absence of exon 78 results in an alternative C-terminus, which may have a function in development (see Chapter 5). Exons 71-74 code for a syntrophin binding domain and the functional meaning of the loss of this site is not yet known. Several transcripts lacking up to 17 exons in the 5' part have been described (Surono 1997 and 1999). Since most of these skips result in an in-frame mRNA, they are thought to generate internally truncated dystrophins that lack the actin-binding domains. The function of these proteins is as yet unknown, and it may be that they are unstable and/or non

functional. Besides the exclusion of exons, one exon has been described that is alternatively inserted into the transcript. Exon 2a is situated in intron 2 and is inserted either between exon 2 and 8 or between exon 2 and 18. These transcripts have been detected in skeletal muscle, small intestine and colon tissue (Pramono 2000). Exon 2a creates a shift in the reading frame. Therefore, the insertion of these exons is more likely to be a naturally occurring artefact (i.e. mistake of the splicing machinery) or an experimental artefact (i.e. cold-shock effect on splicing) than the genuine insertion of an alternative exon. Alternative splicing allows expression of the dystrophins isoforms (see Chapter 5) driven by at least 7 promoters. Three are located before exon 2 (the cortical, muscle and purkinje promoters), whereas the other four are located in intron 29, 44, 55 and 62 (Dp260, Dp 140, Dp116 and Dp71/40, respectively) (Byers 1993; Chelly 1990b; D'Souza 1995; Gorecki 1992; Lederfein 1992; Lidov 1995) (Figure 4 A).
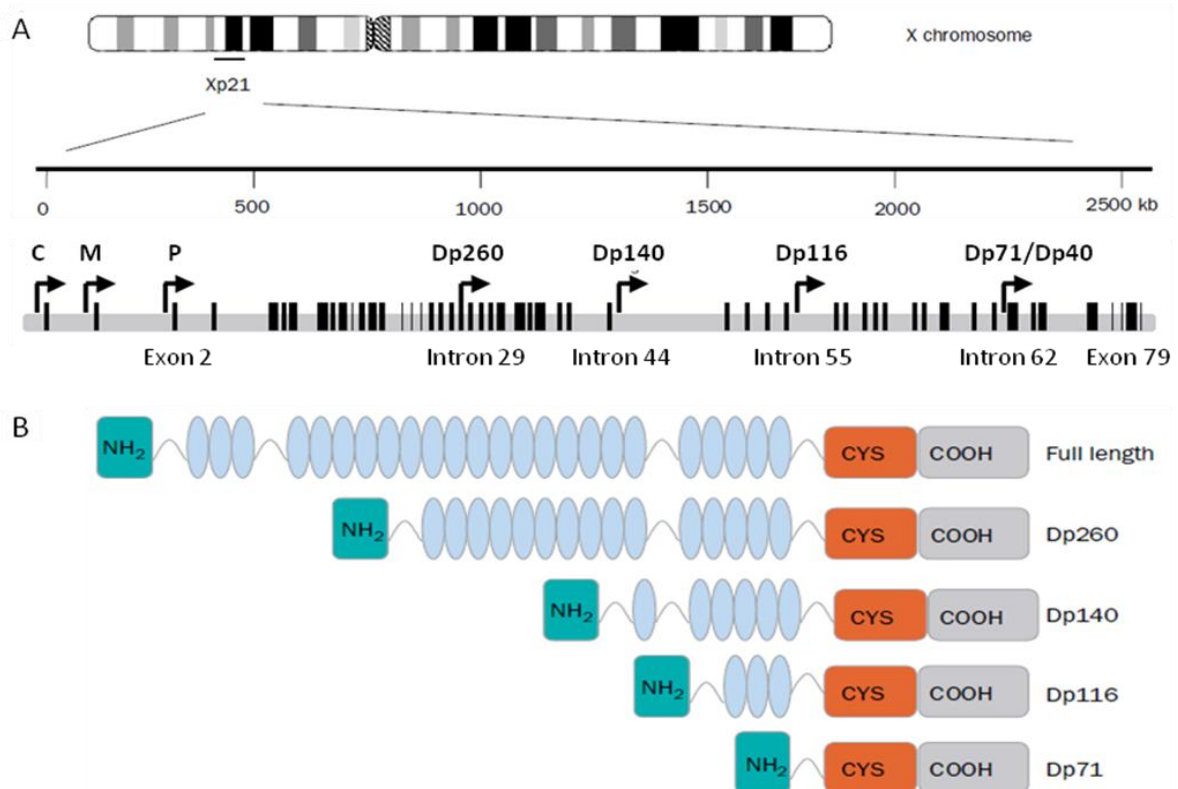

Figure 4. A. Graphic overview of the location of the different promoters for the dystrophin isoforms. The cortical (C), muscle (M) and Purkinje (P) promoters are located proximal to exon 2. The location of the internal promoters is indicated underneath. **B.** Schematic drawing of the different dystrophin isoforms and homologues. The full-length dystrophins Dp427c, Dp427m and Dp427p consist of N-terminal (dark blue), central rod (light blue), cysteine-rich (orange) and C-terminal (violet) domains, but each isoform has its own unique N-terminal part (which is coded by an unique first exon). The shorter isoforms lack some, or most of the N-terminal and/or central rod domains, and also have their own unique first exon (except for Dp140). Dp71 is usually alternatively spliced, which gives rise to an alternative C-terminal part. Dp40 derives from an alternative poly-adenylation signal in intron 70 (Picture kindly provided by dr. Alessandra Ferlini).

Another mysterious aspect of the DMD gene is the final exon, which is 2307 bp long, but only codes for the last 3 amino acids of the dystrophin protein, and the last 31 amino acids for the alternatively spliced isoforms that lack exon 78. Several parts of this large 3' UTR are unusually well-conserved in human, mouse and chicken dystrophin (Greener 2002; Lemaire 1988) and a deletion within the 3' UTR has been described in one BMD patient (Love 1990) and a DMD patient (Spitali 2009). This implies that the UTR may have a regulatory function, although one that as yet remains unknown.

4. Dystrophin and the dystrophin associated Glycoprotein Complex (DGC)

Dystrophin consists of 3685 amino acids and is a 427 kD protein (Koenig 1988). The protein is hydrophilic (31% of the amino acids are charged) and does not have stretches of hydrophobic amino acids, indicating that it is not a transmembrane protein. Dystrophin contains four distinct domains (Figure 4 B). The first 240 N-terminal amino acids define the actin-binding domain, which contains two actin-binding sites (Jarrett 1995; Koenig 1990). This domain is followed by a central rod shaped domain, which consists of 24 spectrin-like repeat units interrupted by 4 proline-rich hinge regions (Koenig 1990). It has recently been shown that repeat units 11-17 contain an additional actin-binding domain (Rybakova 1996). The cysteine-rich domain encompasses amino acids 3080 to 3360 and includes 15 cysteines, two EF hand motifs and a ZZ domain (Koenig 1988). The C-terminal domain consists of the final 325 amino acids, and contains two stretches that are predicted to form α-helical coiled coil domains, which are involved in protein-protein interactions (Koenig 1988). Dystrophin is part of the dystrophin-associated glycoprotein complex (DGC) (Figure 5). The cysteine-rich and C-terminal domains of dystrophin bind to several parts of the DGC (Figure 5 A), which can be divided into the dystroglycan complex, the sarcoglycan-sarcospan complex and the cytoplasmatic, dystrophin containing complex (Blake 2002; Yoshida 1994). In skeletal muscle the dystroglycan complex consists of α-dystroglycan and β-dystroglycan, which are both heavily glycosylated (Ibraghimov-Beskrovnaya 1992). Dystrophin binds to β-dystroglycan, a transmembrane protein that binds to the extra-cellular α-dystroglycan. Alpha-dystroglycan on its part binds to the extracellular matrix component laminin-2 (Hohenester 1999; Rentschler 1999; Suzuki 1994). In this way dystrophin provides a mechanical link between the actin cytoskeleton and the extra-cellular matrix of the

connective tissue. The sarcoglycan-sarcospan complex includes α-, β-, γ- and δ-sarcoglycan and sarcospan (Blake 2002). These transmembrane proteins are hypothesized to have a function in the stabilisation of the dystroglycan-dystrophin complex (Araishi 1999). The cytoplasmatic part of the DGC includes dystrophin itself, α-dystrobrevin and α-syntrophin. Alpha-dystrobrevin binds to both dystrophin and α-syntrophin (Ahn 1996). Alpha-syntrophin also binds to dystrophin and, additionally, it recruits the enzyme nNOS to the sarcolemma (Ahn 1995; Brenman 1996; Brenman 1995; Yoshida 1995).
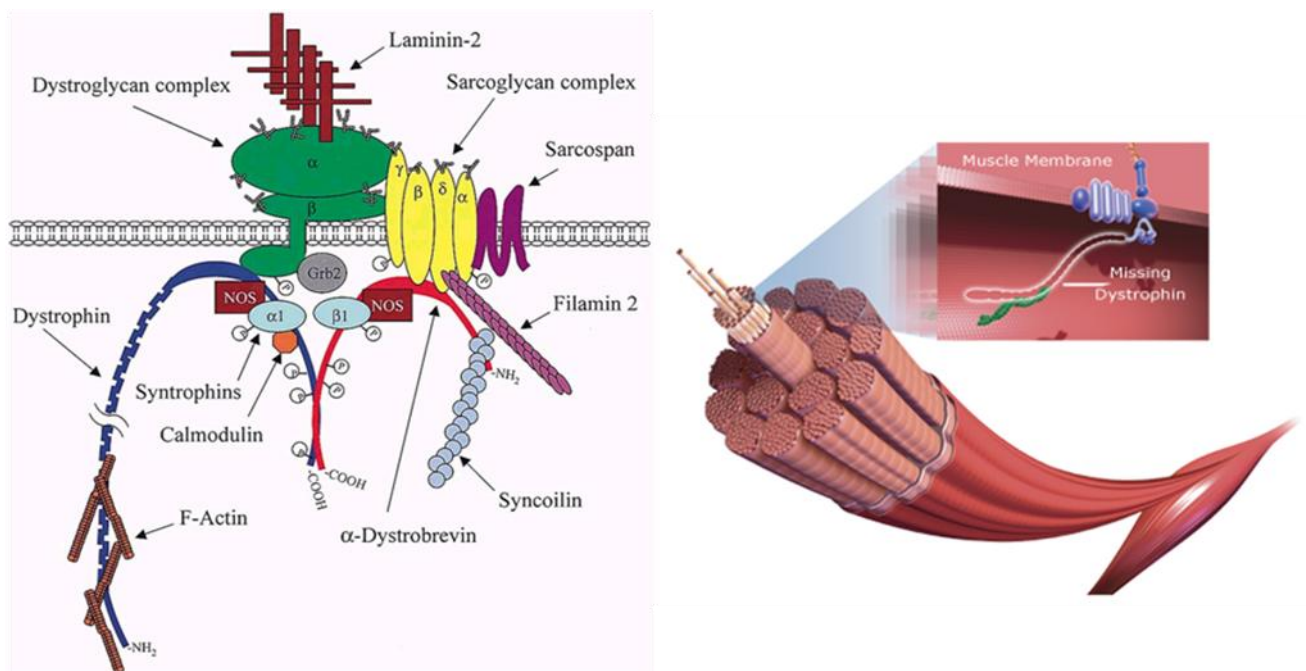

Figure 5. A. Graphic overview of the interactions of the dystrophin protein with the dystrophin associated glycoprotein complex (DGC) at the sarcolemma. **B.** Graphic overview of the correct localization of dystrophin in the myofibers. The figure represents where dytrophin is missing in the DMD patients.

In DMD patients the absence of dystrophin results in the complete loss or decrease of other DGC proteins, and in the loss of nNOS at the sarcolemma (Brenman 1995; Ervasti 1990; Ohlendieck 1991a).

In BMD patients, on the other hand, the internally truncated dystrophins are able to bind to the dystrophin associated proteins, since the C-terminal part of the protein is intact. For most BMD patients the DGC proteins can be detected at the membrane, although in some cases at decreased levels (Matsumura 1994a; Matsumura 1993; Mirabella 1998).

The function of the DGC is still largely unknown. However, since the complex forms a mechanical link between the cytoskeleton and the extracellular matrix, it is assumed that the DGC has a function in maintaining sarcolemma stability during contraction (Matsumura 1994b). Recent studies suggest that the DGC may also have a function in signal transduction, because some DGC proteins can bind to proteins, which are involved in signalling transduction, such as calmodulin, Grb2 and nNOS (reviewed in (Blake 2002; Rando 2001)). Additionally the DGC seems to be required for the functionality of the blood brain barrier, as its permeability is increased and its integrity is lost in mdx mice (the mouse homologue of DMD, see Chapter 8) (Nico 2003; Nico 2004; Vajda 2002). The loss of blood brain barrier function may underlie the mental retardation observed in a portion of DMD patients. The loss of other DGC proteins can also result in muscular dystrophies. Mutations in the α-sarcoglycan gene found in Limb-girdle muscular dystrophy type 2D (LGMD) and in severe childhood autosomal recessive muscular dystrophy (SCARMD) patients (Piccolo 1995; Roberds 1994). Mutations in the other sarcoglycan genes are found in patients suffering from other types of LGMD (Bonnemann 1995; Lim 1995; Nigro 1996; Noguchi 1995). As yet no mutations in the sarcospan or syntrophin genes have been found in patients suffering from muscular dystrophy or other diseases. Moreover, transgenic sarcospan and syntrophin knockout mice do not develop a muscular dystrophy phenotype (Kameya 1999; Lebakken 2000). Dystroglycan knockout mice, on the other hand, are embryonic lethal (Williamson 1997). Recently, mutations in genes coding for fukutin and fukutin related protein have been identified in congenital muscular dystrophy (CMD) and LMGD-2I patients (Brockington 2001b; Kobayashi 1998). These proteins are thought to be glycosyl-transferases, and are likely to be involved in the glycosylation of α-dystroglycan. Indeed, the expression of α-dystroglycan is decreased in CMD and LMGC-2I patients (Brockington 2001a; Hayashi 2001). The exact pathophysiology of DMD is not yet known, but a hypothetical flowchart describes the most important mechanisms thought to give rise to the muscle wasting that is found in DMD patients. Due to the lack of dystrophin and the DGC complex in DMD patients, the muscle membrane is leaky and more vulnerable for mechanical damage (Blake 2002). This results in an influx of calcium into the muscle fiber, leading to a calcium overload of the mitochondrial matrix and the down-regulation of the transcription of mitochondrial genes, resulting in a metabolic crisis and eventually necrosis (Chen 2000). The calcium

influx also over stimulates calcium dependent signal transduction pathways, which eventually causes a downregulation of calcium regulating signal molecules through a negative feedback, enhancing the metabolic crisis (Chen 2000). Finally, the calcium influx activates calcium dependent proteases in general, and the calpaines in particular (Alderton 2000; Spencer 1995). The proteases break down the muscle membrane and modify calcium leak channels, thus augmenting the influx of calcium into the cell (Alderton 2000). Due to the muscle damage the fiber becomes "leaky" and muscle enzymes such as creatin kinase will leak into the bloodstream. Additionally, since nNOS synthesizes the molecule NO that induces vaso-dilatation, the absence of nNOS can cause ischemia after severe mechanical stress. This can lead to necrosis (Sander 2000, Thomas, 1998), which attracts inflammatory cells, such as dendritical cells, mast cells and T-cells, which eventually trigger fibrosis (McDouall 1990; Nahirney 1997).

5. Dystrophin isoforms and homologs

Besides the dystrophin initially detected in skeletal muscle and cardiomyocytes, at least seven promoters drive the expression of other dystrophin isoforms from the DMD gene (Figure 4 B, Table 1). In addition to the muscle dystrophin – named Dp427m, because of its molecular weight (427 kD) and its main site of expression (muscle) – two other full-length dystrophins are produced, i.e. Dp427c and Dp427p (Boyce 1991; Chelly 1990b; Gorecki 1992; Holder 1996). Both isoforms have their own unique first exon, which is spliced to exon two of the Dp427m transcript (Boyce 1991; Gorecki 1992). Dp427c is mainly expressed in the cortical neurons – hence the postfix 'c' – and the hippocampus in the brain (Barnea 1990; Gorecki 1992), whereas Dp427p is expressed in the cerebellar Purkinje cells and at lower levels in skeletal muscle (Gorecki 1992; Holder 1996). There are also four internal promoters from which shorter isoforms are produced; (Byers 1993; D'Souza 1995; Hugnot 1992; Lidov 1995) named after their respective molecular weights. Dp260 is expressed in the retina and is derived from a unique first exon, located in intron 29, which is spliced to exon 30 of Dp427m (D'Souza 1995). Two Dp260 isoforms (Dp260-1 and Dp260-2) are formed by alternative splicing of the first exon and a different translation initiation site. Dp260 lacks the N-terminal domain, two hinge regions and nine repeats, when compared to the fulllength dystrophins. The promoter of Dp140 is located in intron 44 (Lidov 1995). The methionine translation

initiation codon, however, is located in exon 51, and as a consequence this protein does contain a large 5' untranslated region (UTR), but no isoform specific amino acids. Dp140 has retained only the last two hinge regions, five of the spectrin-like repeats, the cysteine-rich and C-terminal domain. The protein is found throughout the central nervous system (Lidov 1995). Dp116 has a unique N-terminal part, which is coded by an exon located in intron 55. This exon is spliced to exon 56 of Dp427m, which codes for the distal part of the 21st repeat unit (Byers 1993). This isoform is expressed in Schwann cells. Dp71 and Dp40 derive from a unique first exon in intron 62 that is spliced to exon 63 (Blake 1992; Feener 1989; Hugnot 1992; Lederfein 1992; Tinsley 1993). Both proteins have an alternative C-terminal domain when compared to the other dystrophin isoforms. In Dp71 exon 78 is often omitted, resulting in a shifted reading frame and the insertion of 31 new amino acids instead of the original final 13 amino acids (Lederfein 1992). These amino acids are mainly hydrophobic, whereas the original C-terminal part is hydrophilic. Dp40 uses an alternative poly adenylation site located in intron 70, which results in a shortened C-terminal domain (Feener 1989; Tinsley 1993). Both isoforms are ubiquitously expressed in non-muscle cells (Hugnot 1992; Tinsley 1993). The diversity of dystrophin isoforms is further increased by alternative splicing of the 3' part of the gene (Feener 1989) (see Chapter 3). As yet the functional significance of the different dystrophins is unknown. It is assumed that the full-length isoforms will have a comparable function to Dp427m, i.e. providing a mechanical link between the cytoskeleton and the extracellular compartments. Since the shorter isoforms do not contain an actin-binding domain, these dystrophins can only bind to the DGC proteins and are thought to be involved in the stabilisation and function of non-muscle DGC-like protein complexes (Blake 2002). Dp260 is thought to be required for normal retina function, since this isoform is expressed mainly in the retina and some DMD patients have abnormal electro-retinograms (D'Souza 1995; Pillers 1993). The hydrophobic Dp71 has been detected in embryonic tissues and in developing muscles (Howard 1999; Sarig 1999). It is replaced by the hydrophilic Dp427 when the muscle cells mature, indicating that it may have a function in development (Howard 1999). The dystrophin isoforms may also have a function in signal transduction, either by binding to signalling molecules themselves or by recruiting proteins that have a function in signalling transduction (reviewed in (Rando 2000)). Dp427c, Dp427p, Dp140 and Dp116 are expressed in brain and the central nervous

system. As a result of the mutation one or more isoforms may be absent in addition to the Dp427m isoform, and cause the mental impairment sometimes found in DMD and BMD patients (Byers 1993; Comi 1995; Gorecki 1992; Lidov 1995). In some studies a correlation has been found between the absence of Dp140 (due to mutations spanning the Dp140 promoter) and the presence of mental abnormalities in DMD and BMD patients (Bardoni 2000; Bushby 1995; Felisari 2000). A very recent study shows the correlation between the Full Scale Intelligence Quotients (FSIQ) results with the location of the dystrophin gene mutation suggesting that the risk of cognitive deficit is a result of the cumulative loss of central nervous system (CNS) expressed dystrophin isoforms, and that correct classification of isoform involvement results in improved estimates of risk (Taylor 2010).

Table 1. Characteristics of the different dystrophin isoforms.

Isoform	Molecular Weight	mRNA	Main site of expression
Dp427c	427 KDa	1st exon spliced to exon 2	Cortical neurons and hippocampus
Dp427m	427 KDa	1st exon spliced to exon 2	Skeletal muscle and cardiomyocytes
Dp427p	427 KDa	1st exon spliced to exon 2	Cerebellar purkinje cells
Dp260	260 KDa	1st exon spliced to exon 30	Retina
Dp140	140 KDa	1st exon spliced to exon 45	Central nervous system
Dp116	116 KDa	1st exon spliced to exon 56	Schwann cells
Dp71	71 KDa	1st exon spliced to exon 63	Ubiquitous
Dp40	40 KDa	1st exon spliced to exon 63	Ubiquitous

In addition to the isoforms derived from the DMD gene, there are also three known dystrophin homologues, which are derived from other genes: utrophin, dystrophin related protein 2 (DRP2) and dystrobrevin. Utrophin (also called dystrophin related protein 1 (DRP1)) shares considerable homology with dystrophin along the entire length on both DNA and protein level (Tinsley 1992).

The utrophin gene is located on chromosome 6 and consists of 74 exons dispersed over 0.9 Mb (compared to 2.4 Mb for dystrophin) (Pozzoli 2002). The 13 kb mRNA gives rise to a 395 kDa protein (Tinsley 1992), which contains an N-terminal actin binding

domain, a central rod domain, a cysteine-rich and C-terminal domain (Tinsley 1992). When compared to dystrophin, utrophin lacks repeat units 15 and 19 and hinge regions 1 and 3 (Winder 1995a), and the N-terminal domain of utrophin has a higher affinity for actin (Winder 1995b). Utrophin is ubiquitously expressed (hence the name: ubiquitously expressed dystrophin-like protein (Blake 1992)). In muscle fibers utrophin is expressed in the neuromuscular synapses and the myotendinous junctions, where it participates in post-synaptic membrane maintenance and acetylecholine receptor clustering (Nguyen 1991). In vitro and in vivo studies have shown that utrophin can bind to β-dystroglycan, α-dystrobrevin, syntrophin and the sarcoglycans and it is assumed that utrophin forms a DGC-like complex (Matsumura 1992; Peters 1998). In developing and regenerating muscle, utrophin is upregulated and present along the entire sarcolemma (Pons 1993). This is also observed in DMD patients (Galvagni 2002; Karpati 1993; Mizuno 1993; Mizuno 1994), suggesting that there is some functional redundancy between dystrophin and utrophin (Blake 2002). However, the utrophin overexpression found in DMD patients is apparently too low to fully prevent muscle degeneration (upregulation of the utrophin gene as a therapeutic strategy for DMD is discussed in Chapter 10).

The dystrophin related protein 2 (DRP2) shows similarity to Dp116. It has a unique proline-rich N-terminal part, followed by two spectrin-like repeats, a cysteine rich domain and a C-terminal domain, which are homologous to those of dystrophin (Roberts 1996). DRP2 is not expressed in muscle, but is expressed in brain where it is associated with the postsynaptic densities and cholinergic neurons (Roberts 2000). The α- and β-dystrobrevin proteins are derived from two different genes and both show homology to the C-terminal part of dystrophin (Blake 1998; Sadoulet-Puccio 1996; Wagner 1993). At least five different α-dystrobrevin isoforms are generated through the use of alternative promoters and alternative splicing (Blake 1996; Sadoulet-Puccio 1996). These isoforms can bind to dystrophin and are located at the neuromuscular junction (Nawrotzki 1998; Peters 1998). Beta-dystrobrevin is expressed in non-muscle cells, and is complexed with utrophin and Dp71 (Blake 1999; Loh 2000). Dystrobrevins contain a domain that can be phosphorylated by tyrosine kinases, and can associate with several proteins involved in signalling transduction (Blake 2002).

6. Mutations

The mutation rate for the DMD coding sequence is estimated at 1×10^{-4} genes per generation (Blake 2002). This is high compared to an estimated average mutation rate of $1 \times 10^{-5} - 10^{-6}$ for human genes (Lewin 2000). As a consequence of this relatively high mutation rate, one third of all mutations are de novo (Laing 1993), and there is a broad variation of different mutations. Intragenic deletions of one or more exons are found in ~65% of all patients (Koenig 1989; White 2002), while duplications are found in 6-8% of all patients (Galvagni 1994; White 2002). Nonsense or frame-shifting mutations in the DMD gene lead to Duchenne muscular dystrophy (DMD) (MIM# 310200), by contrast, Becker muscular dystrophy (BMD)(MIM# 310376) is caused by in-frame mutations that give rise to a smaller but functional protein (Hoffman 1987; Monaco 1988). The interruption or maintenance of the dystrophin reading frame by the gene mutations explains the phenotypic differences observed in approximately 92% of the BMD/DMD cases (Aartsma-Rus 2006a; Koenig 1989). The majority of the deletions and duplications cluster into two hotspot regions: the minor hotspot spans exons 2-20 and the major hotspot region exons 45-53 (Beggs 1990; Liechti-Gallati 1989). The highest numbers of breakpoints are found in the extremely large introns 1, 2 and 7 (major duplication and minor deletion hotspots) and 44 (major deletion hotspot) (Tuffery-Giraud 2009; Fokkema 2005). However, if you correct for the length of the introns (i.e. calculate the number of breakpoints per kb intron), relatively high numbers of breakpoints are located in introns 45 trough 53. There is as yet no explanation for the preferential occurrence of breakpoints in these introns. Extensive analysis of 22 breakpoints in intron 49 revealed that the breakpoints were evenly spread throughout the intron, and a sequence motive near or at the breakpoints could not be identified (Nobile 2002). Further research of breakpoints in introns 47 and 48 showed that most breakpoints cluster around intronic motifs that could predispose for double-stranded DNA breaks (Sironi 2003; Toffolatti 2002). However, these motives are present throughout the gene and not only in intron 47 and 48, so there must be another underlying mechanism for the predisposition of breakpoints in the two hotspots (Sironi 2003). Another explanation is that the breakpoints are in fact evenly spread throughout the gene, but that we observe the hot spots due to the fact that only breakpoints occurring in the hot spots result in a phenotype. This may hold true for breakpoints in introns 22 through 40. A deletion of this region (that contains only in-frame exons) is usually associated with a

very mild phenotype. However, even in-frame deletions in the proximal introns can result in a DMD phenotype (Flanigan 2009).

In some cases a mutation detected in affected siblings, cannot be found in the somatic cells of their mother, suggesting that the mutation is only present in part of the germ-line cells of the mother (Bakker 1987). The estimated frequency of this so-called germ-line mosaicism within a family varies between 12 and 20% (Emery 1995). Unexpectedly, it has been shown that deletions found in children from germline mosaic carriers involve the proximal part of the gene in 79% of all cases, while mutations in the distal part were found in only 21% (Passos-Bueno 1992). The reason for this phenomenon is as yet unknown.

For diagnostic purposes the high frequency of deletions clustering in the hotspots is an advantage; 98% of all DMD/BMD deletions can be detected using multiplex PCR of only 8 exons (Beggs 1990; Chamberlain 1988). Southern blot analysis, multiplex amplifiable probe hybridisation (MAPH), Multiplex ligation-dependent probe amplification (MLPA), high resolution melting curve analysis (MCA) and comparative genomic hybridization array (CGH) can be used to detect the precise range of both deletions and duplications (Hodgson 1992; White 2002; Janssen 2005; Almomani 2009; Hegde 2008; Saillour 2008; del Gaudio 2008; Bovolenta 2008). In about one third of the patients small deletions and insertions or point mutations are found (Deburgrave 2007). These mutations are evenly spread throughout the gene and are therefore harder to find (Flanigan 2009). They can be detected by denaturant gradient gel electrophoresis (DGGE), single strand conformation polymorphism analysis (SSCA), direct sequencing or chemical cleavage of each individual exon, the protein trunction test (PTT), or by single-condition amplification/internal primer (SCAIP) (Dolinsky 2002; Flanigan 2003; Hofstra 2004; Roest 1993, Kilimann, 1992; Tuffery 1993; Tuffery-Giraud 1999; Spitali 2009; Flanigan 2009). The majority of the detected mutations results in-frameshifts, non-sense codons, the abolishment of splice sites, or the introduction of cryptic splice sites (Kilimann 1992; Tuffery-Giraud 1999; Flanigan 2009). DMD causing missense mutations account only for 0,18% of all mutations (Flanigan 2009) and were located in an actin-binding domain or in the part of the cysteine rich region that binds to β-dystroglycan (Goldberg 1998; Lenk 1996; Prior 1995; Flanigan 2009).

The reading frame rule holds true for over 93% of all BMD and DMD patients (Aartsma-Rus 2006a), however exception to the rules are found expecially for 5' gene deletions occurring in BMD patients, a region known to be hotspot for exceptions (Kesari 2008).

For in-frame mutations the phenotype is also to some extent determined by the location and the size of the deletion (Arikawa-Hirasawa 1995; Beggs 1991; Fanin 1996). Huge in-frame deletions removing up to 35 exons in the central rod domain have been described for relatively mild Becker patients (England 1990; Fanin 1996; Mirabella 1998). However, larger deletions are always associated with a DMD phenotype, suggesting that at least a small part of the rod domain is required for proper dystrophin function (Fanin 1996). Deletions removing only the first part of the central rod domain have been found in a-symptomatic individuals, in persons with elevated CK levels and in patients suffering from muscle cramps upon exercise (Angelini 1994a; Gospe 1989; Ishigaki 1996). In-frame deletions in the major hotspot generally cause typical BMD, while deletions in the actin-binding domain cause severe BMD (Beggs 1991). Deletions removing both the actin-binding domain and part of the central rod domain usually cause DMD (Arikawa-Hirasawa 1995; Fanin 1996; Vainzof 1993). This may be explained by the fact that a third actin-binding site is present in the central rod domain (Rybakova 1996). The removal of the first two actin-binding domains (located in the actin-binding domain) may partially be compensated for by this central rod actin-binding domain and therefore cause severe BMD, whereas the deletion of all three domains results in DMD. Deletions in the C-terminal domain are usually associated with DMD (Bies 1992). In addition to the location of the deletion, the amount of dystrophin produced in the patients also correlates with the phenotype: the more dystrophin a patient produces, the milder the phenotype (Angelini 1994a; Hoffman 1988). No dystrophin or up to 3% of normal levels are usually found in DMD patients, whereas levels of over 30% of dystrophin (of an abnormal weight) are found in BMD patients (Neri 2007).

Certain in-frame mutations are always associated with a DMD phenotype, whereas a number of out-of-frame mutations lead to a BMD phenotype (Fokkema 2005). In-frame mutations may be too large to result in a functional dystrophin or they may be located in areas of the protein, which are indispensable (see above). However, for a significant part of in-frame DMD patients no dystrophin protein can be detected. This may be because additional exons are be spliced out as a consequence of the deletion, resulting

in out-of-frame transcripts. Alternatively, the in-frame deletion could lead to an unstable dystrophin. Out-of-frame mutations on DNA level associated with a BMD phenotype do not necessarily have to be inconsistent with the reading frame rule on RNA level. For instance, two nonsense mutations in the in-frame exon 29 disrupt a sequence that is required for the proper inclusion of this exon (Ginjaar 2000; Franz 2000). As a result, exon 29 (and thus the truncating mutation) is not always included in the transcript by the splicing machinery. Dystrophin lacking the amino acids coded by exon 29 can be detected in these patients, explaining why they have a BMD and not a DMD phenotype. Unfortunately, for the majority of all patients mutations are determined on DNA level only, and not confirmed on RNA level. Therefore, it is not known whether apparent inconsistencies with the reading frame rule are also present on RNA level. There are also a number of deletions (detected on DNA level), which are found in both DMD and BMD patients. The majority of these mutations start either in intron 3 or in intron 44. Exon 44 skipping in patients with a deletion of exon 45 (Prior 1997; Roberts 1991), or a duplication of exon 44 (Aartsma-Rus personal communication) has been described, and this might explain the discrepancy: high levels of alternative splicing of exon 44 will generate in-frame transcripts and internally deleted dystrophins. These will therefore be associated with a BMD phenotype, while no or low levels of exon 44 skipping will result a DMD phenotype. The levels of alternative splicing are influenced by the different splicing regulation patterns of each individual and will additionally be influenced by the extent of the deletions and the location of the breakpoints in intron 44. Even though the deletions may seem identical on DNA levels, in reality the exact location of the breakpoints differs between individuals of different families. However, as yet no intronic regulating sequences that influence the inclusion or exclusion of exon 44 have been identified. The deletion of exon 3-7 is the most commonly found and most extensively investigated deletion that is associated with both phenotypes. Alternative splicing of exon 2 would restore the reading frame for these patients and has been described for two BMD patients albeit at levels of 1-2% (Chelly 1990a). Despite extensive research exon 2 skipping has not been described elsewhere for patients with this deletion (Gangopadhyay 1992; Winnard 1993; Winnard 1995). An explanation for this phenomenon may be the use of an alternative translation initiation codon (Winnard 1995). This hypothesis is supported by the fact that there are three in-frame ATG codons present in exon 8. Furthermore, the dystrophins detected in these BMD patients

where shown to lack the exon 1 and 2 encoded sequence, but did contain the exon 8 encoded sequence (Winnard 1995). In addition to BMD and DMD, mutations in the DMD gene have also been found in X-linked dilated cardiomyopathy (XLDC) patients (Muntoni 1993). These patients have no signs of skeletal myopathy but suffer from rapidly progressive congestive heart failure (Milasin 1996). Without heart transplantation premature death usually occurs within 2 years after diagnosis (often in their twenties).

Most mutations are present in the first exon of the muscle specific dystrophin isoform and completely abolish dystrophin expression in the heart (Ferlini 1999, Neri 2007). In skeletal muscle, on the other hand, upregulation of the purkinje and brain isoforms rescues the phenotype (Bastianutto 2001). This upregulation is controlled by the dystrophin muscle enhancer 1 that is located downstream of the muscle specific first exon, and which increases the expression of the Dp427c and Dp427p isoforms exclusively in skeletal muscle cells. In-frame deletions involving exon 48 and exon 49-51 have been detected in less severely affected XLCD patients, and in BMD patients (with or without XLDC) (Ferlini 1999 and Ferlini personal communication).

7. Revertant Fibres

Individual dystrophin-positive fibers have been found in DMD patients and in otherwise dystrophin-negative mdx mice (see Chapter 8) (Thanh 1995; van Deutekom 2007; Rimessi 2009) (Figure 6). These 'revertant fibers' grow in clusters and comprise 1% to 10% of all fibers. The frequency of these fibers increases with age in both DMD patients (Fanin 1995) and mdx mice (Lu 2000), and after mutagene doses of X-rays in mdx mice (Hoffman 1990). The revertant dystrophins are located at the membrane and may be functional. The correlation between the amount of revertant fibers and the severity of the DMD phenotype is still controversial (Fanin 1995; Nicholson 1993; Thanh 1995). Revertant dystrophins were shown to lack domains coded by the exons adjacent to exons deleted in the DMD patients (Fanin 1995; Thanh 1995). By omitting these exons the reading frame was restored, explaining why BMD-like, probably semi-functional dystrophins could be produced. Revertant fibers have been studied more extensively in mdx mice (Lu 2000). A few isolated dystrophin positive fibers were detected in newborn mice and the amount of revertant fibers, the number of fibers per cluster and the length of the fibers increased with age (2 weeks to 18 months). Not only was the dystrophin

synthesis restored in revertant fibers, dystrophin and the DGC-complex were also correctly located at the membrane, providing more evidence that the revertant dystrophin is at least partly functional. Different inframe transcripts lacking multiple exons were identified in different clusters. In most dystrophins only domains immediately surrounding the mutated exon 23 were missing.
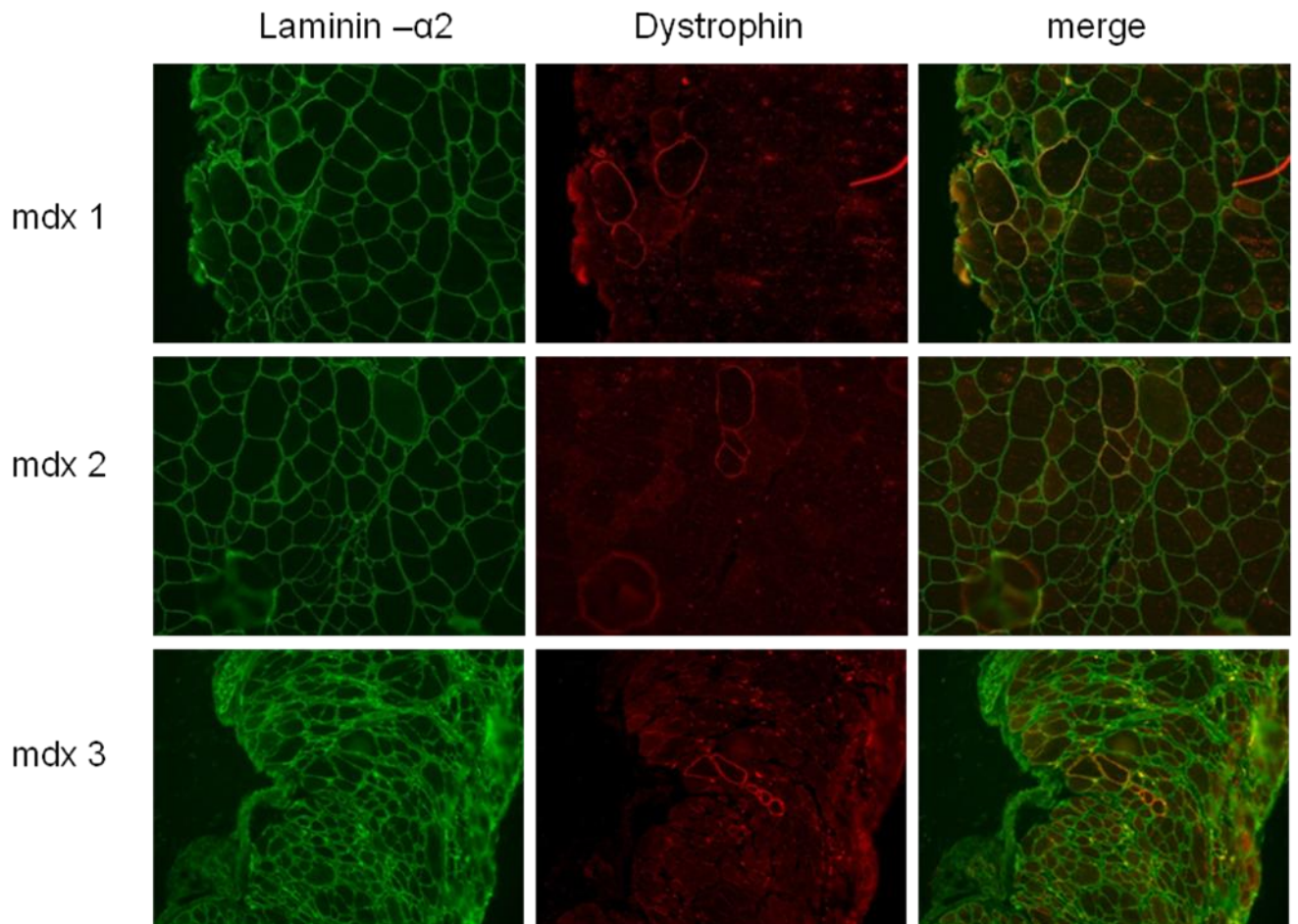

Figure 6. Immunofluorescence analysis performed on mdx muscle cross sections reveals the presence of revertant fibers. Laminin-α2 (green) and dystrophin (red) colocalize at the sarcolemma of the myofibers (Pictures kindly provided by Elena Bassi).

However, dystrophins lacking up to 35 exons were also detected. Potential mechanisms responsible for the occurrence of revertant fibers are somatic reversion and alternative splicing (Klein 1992; Lu 2000). There are arguments in favour of, and against, both mechanisms. Somatic reversion, i.e. the occurrence of secondary, frame-restoring mutations in muscle or muscle precursor cells, fits with the clonal nature of the revertant fibers, their increase with age and their appearance after exposure to mutagenic doses of X-rays. Conversely, mutations are generally proliferation-related events and would be

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more likely to occur during the many cell divisions in the embryogenesis than in differentiated muscles. However, only very low levels of revertant fibers are found in newborn mice. Furthermore, in situ hybridisation experiments were not sensitive enough to detect deletions on DNA level with a probe for intron 22 in mdx revertants (the probe detected 80% positive nuclei in both dystrophin positive and dystrophin negative fibers). This means that there is either no deletion present or that the deletion is present in too low a number of nuclei to detect a significant difference. This finding, however, does not exclude the possibility of a point mutation in a splice site. In fact, it was recently shown that the substitution of one base pair in the acceptor splice site of exon 23 induces several different alternative splicing patterns that involve 12 or more exons (Bertoni 2003). The other potential mechanism, alternative splicing, has been described for the DMD gene throughout the coding sequence in both patients and unaffected individuals (Sironi 2002). Furthermore, in a substantial part of revertant fibers investigated in mdx mice, two non-consecutive domains were absent in the revertant dystrophins, located around exon 23 and around exon 40-46 (Lu 2000). Intuitively one would think this to be more likely the result of two independent alternative splicing events than of two different new mutations. It should be noted, however, that deletions of two non-consecutive parts of the DMD gene have been reported for DMD patients (Zatz 1998). Finally, one should also not overlook the possibility of a combination of the two mechanisms. Mutations frequently occur in the mutation hotspot located between exon 45-55 and alternative splicing around exon 23 has been observed both in normal and mdx mice (personal observation).

Alternative splicing seems to be at odds with the clonal nature of the fibers. Lu and colleagues proposed that the splicing pattern is changed during degeneration and regeneration and that a pattern of frame-restoring splicing is preserved by functional selection (Lu 2000). The revertant dystrophin would protect the fiber from degeneration and the "splicing imprinting" would expand to adjacent fibers, thus explaining the clonal nature of revertant clusters. However, this hypothetical mechanism has never been demonstrated.

8. Animal models

Several animal models carrying mutations in the DMD gene were reported, among these the mdx mouse, the double knockout mdx/utrn$^{-/-}$ mice and the golden retriever muscular dystrophy (GRMD) dog are used most.

The mdx mouse is a naturally occurring dystrophin-deficient mutant, which was first described in 1984 in a colony of C57BL/10 mice (C57BL/10ScSnJ) (Bulfield 1984) and it is now called C57BL/10ScSn-Dmd^{mdx}/J; it is readily available from commercial breeders and thus widely used in basic and translational research. It carries a point mutation in exon 23 of the mouse dystrophin gene introducing a premature stop codon, which leads to the absence of full-length dystrophin. Mdx mice have a slightly shorter life span as compared to wildtype controls (Chamberlain 2007). The muscle degeneration in mdx mice differs from DMD patients in that it appears in waves and is not a continuum. At first, a marked degeneration and regeneration of muscle fibers is observed at a young age (2–4 weeks (McGeachie 1993)), which results in an increase in the number of newly differentiating myofibers characterized by centralized nuclei and an increased heterogeneity in myofiber size. In parallel, necrosis is observed in muscles at this early stage (Grounds 2004; Messina 2006) while it decreases again after 60 days (Spencer 1996). Subsequently, loss of muscle tissue is slow and general muscle weakness in cage-reared animals is not evident until later in life (Lefaucheur 1995). Fibrosis in most mdx mouse muscles is less pronounced than in DMD patients with the exception of the diaphragm muscle (Stedman 1991; Connolly 2001; Rolland 2006). Although absolute muscle force of limb muscles remains comparable to unaffected mice, the relative force normalized to body weight decreases by approximately 20% and 50% at the age of 8–10 weeks and 4 months, respectively (Messina 2006; Connolly 2001; Raymackers 2003). However, others could not confirm this finding (Burdi 2006; Frysse 2004), which is probably based on variations in the mice strains (e.g. body weight, cooperation of the animals in force measurement assays) and on different experimental procedures. Similarly, a reduced force of contraction has also been reported in isolated muscle preparations although the lack of standardized conditions makes it again difficult to compare the results obtained by different groups (Deconinck 1998; Lynch 2001; Louis 2004; Laws 2004). These discrepancies clearly show that the availability of standardized experimental protocols would be of advantage both to elucidate basic

aspects of the pathology and to test emerging therapeutic strategies. Respiratory pathology is evident in 16-month-old mice but not in 4-month-old mice, showing a worsening of respiratory capacity with age, in line with the increased histological damage of respiratory muscles (Gayraud 2007). Abnormal cardiac function is also seen in the mdx mouse sharing several characteristics of DMD-associated cardiomyopathy (Quinlan 2004). For example, changes in the electrocardiogram (ECG) and echocardiogram have been reported in mice that are older than 6 months (Bia 1999; Wehling-Henricks 2005; Spurney 2008). This change in cardiac function is paralleled by an increase in myocardial fibrosis and the occurrence of foci of myocardial necrosis and inflammation (Quinlan 2004; Van Erp 2006; Cohn 2007; Buyse 2009). Mdx mice have served as a model for several studies on calcium homeostasis and alterations thereof. Basal cytosolic calcium levels were reported to be identical in mdx and wild-type mice (De Backer 2002) showing a very robust calcium homeostasis, possibly due to elevated expression of the calcium-buffer parvalbumin (Gailly 1993; Sano 1990). Nevertheless, [Ca2+] in the subsarcolemmal compartment (Mallouk 2000) and in the sarcoplasmic reticulum (Robert 2001) was increased and levels of inositol 1,4,5-triphosphate (IP3) and of the IP3 receptor were higher in mouse and human dystrophic cell lines (Liberona 1998). Muscle pathology in the mdx mouse can be aggravated by forced exercise, a method introduced to allow a better evaluation of the efficacy of experimental therapies. Several exercise paradigms have been described (Granchelli 2000; Vilquin 1998; Hodgetts 2006). Muscle damage of exercised mdx animals is increased as reflected by an increased number of muscle fibers with centralized nuclei and increased fibrosis (Burdi 2006; De Luca 2005). Plasma creatine kinase levels are further elevated, calcium homeostasis is more severely impaired and fore-limb strength is generally reduced (Rolland 2006; Burdi 2006; Fraysse 2004; De Luca 2005) while body weight remains unchanged. Cardiomyopathy has been described for the exercised mdx mouse (Nakamura 2002), however, a systematic analysis of the impact of exercise on cardiac pathology is still lacking.

In an attempt to aggravate the pathology of the mdx model and to study utrophin-based gene therapy strategies, utrophin mutant mice (on C57B5/129J background) were crossed with C57BL/10 mdx mice to obtain double knock-out mice (Deconinck 1997; Grady 1997). Mice lacking both dystrophin and utrophin share many phenotypical hallmarks with DMD. The mice have a markedly reduced life span of 4–20 weeks,

severe muscle weakness with joint contractures, kyphosis and a pronounced growth retardation that starts around weaning. Necrotic fibers and connective tissue are visible in the diaphragm already 6 days after birth and centrally nucleated fibers are evident at 2 weeks. Interestingly, the diaphragm is not more affected than that of the mdx mice, suggesting that utrophin does not compensate for the lack of dystrophin in this muscle (Deconinck 1997; Grady 1997). The skeletal muscles appear normal at birth but show first signs of degeneration/regeneration already after 2 weeks. Although the dystrophic pathology is similar to mdx mice at 4–5 weeks of age, it then remains severe in contrast to the improvements seen in mdx mice. By 10 weeks of age, fibrosis is evident in tibialis anterior muscle. Occasionally, larger necrotic areas are observed in the heart (Grady 1997). The neuromuscular and the myotendinous junctions in mdx/utr$^{-/-}$ mice show a marked reduction in membrane folds, presumably as a result of the lack of utrophin (Deconinck 1997; Grady 1997). Therefore, both the disease progression and the phenotype of these double mutant mice resemble the progression of muscle dystrophy in DMD patients and the interstitial fibrosis of skeletal muscle lends itself to assess the efficacy of anti-fibrotic treatments.

In the golden retriever, the best characterized dog model, the disease results from a single base pair change in the 30 consensus splice site of intron 6, leading to skipping of exon 7 and alteration of the reading frame in exon 8, which creates a premature stop codon (Sharp 1992). Unlike the dystrophin-deficient mdx mouse, GRMD dogs suffer from a rapidly progressing, fatal disease similar to DMD. There is, however, a large variation in disease severity as some pups survive only for a few days, while others are ambulant for months or even years (Ambrosio 2008). To account for this variation, it is possible to distinguish between the severe and the less severe pathology (Ruegg 2009). Incomplete muscle repair in GRMD results in progressive weakness and gait abnormalities at the age of 6–9 weeks leading to muscle atrophy, fibrosis and contractures by 6 months (Ruegg 2009). The growth of affected pups is delayed and their walking ability is impaired. The dogs become less active than non-affected littermates at about 9 months of age. At this time, their gait is still uncoordinated and their limbs are abducted (Valentine 1988). Severely affected dogs have difficulties to rise and can walk only a few steps. At the age of 12 months, pharyngeal and esophageal dysfunctions start to develop and respiratory capacity is decreased. Kyphosis develops by the 6th month of age. Moreover, as seen in DMD patients, GRMD

dogs display selective muscle involvement although the most affected muscles in dogs (i.e. tongue, masticatory and trunk muscles) are different to those of humans (Kornegay 2003). Myocardial involvement is much more evident in the golden retriever than in other animal models, matching very closely the cardiac complications encountered in DMD patients. Atrophy already appears during the neonatal period (Valentine 1988), although histological changes are generally not present up to 3 months of age (Moise 1991). Systolic dysfunction and left ventricular dilation becomes apparent after several months or years (Moise 1991), although Tissue Doppler Imaging has allowed the detection of severely impaired systolic and diastolic indices in young dogs (25 ± 11 weeks) (Chetboul 2004). Recently, NMR techniques have been applied to assess differences between dystrophic and healthy muscles in the dog, a method that may be useful to monitor a potential therapeutic response (Thibaud 2007).

Other animal models are the HFMD cat (Gaschen 1999), the mdx 2CV-5CV mice (Chapman 1989), the mdx52, mdx/myoD^{-/-}, mdx/adbn^{-/-}, mdx/a7 integrin^{-/-}, mdx/PV^{-/-}, the Beagle (CXMDJ) (Ruegg 2009) and the Cavalier King Charles Spaniels dogs (Walmsley 2010).

Finally a mouse carrying the entire human DMD gene on a yeast artificial chromosome (YAC) was generated (hDMD mouse) ('t Hoen 2008). RT-PCR, Western blot and immuno-histochemical analyses confirmed the expression of the human dystrophin in muscle, heart, kidney and testis. In the mdx mouse, the human dystrophin was able to rescue the dystrophic phenotype ('t Hoen 2008). This system allows for the testing of genetic therapies affecting the human gene in a mouse background.

9. Current therapies

Pharmacological Treatment with Corticosteroids

Glucocorticoid administration is currently the only drug treatment known to offer real clinical benefit to patients suffering from Duchenne muscular dystrophy (DMD). Glucocorticoids used to treat DMD include prednisone (Manzur 2004) and its oxazoline derivative, deflazacort (de Groot 2006). DMD patients treated with glucocorticoids exhibit delayed progression of muscle weakness (Mendell 1989) and remain ambulatory for a greater period of their lives (Griggs 1991). Different doses as well as different

intermittent schemes of administration have been tried in clinical trials with the aim of reducing side effects (Angelini 1994b; Merlini 2003; Biggar 2004; Connolly 2002; Beenakker 2005). However, there is as yet no internationally accepted consensus on which corticosteroid treatment scheme is the best for patients with DMD. In addition, there has yet to be a randomized study involving a head-to-head comparison. The mechanism by which patients benefit from glucocorticoid treatment is not fully understood, although it is thought that the clinical benefits arise in part from the anti-inflammatory and immunosuppressive effects of these drugs (Tidball 2004). St Pierre and colleagues have shown that deflazacort treatment of the mdx mouse can alleviate symptoms of the dystrophic pathology and results in the stimulation of utrophin A expression in skeletal muscle fibers (St Pierre 2004) via an IRES element located at the utorphin 5'UTR (Miura 2008).

10. Potential Treatments

Pharmacological Treatments

Utrophin Upregulation

The utrophin gene spans 1 Mb, giving rise to a 13 kb cDNA and a 400 kD protein (Pozzoli 2002). Dystrophin and utrophin show over 80% similarity in the actin-binding, cysteine-rich and C-terminal domains, and 35% similarity in the central rod domain (Pearce 1993; Tinsley 1992). Utrophin is ubiquitoussly expressed, but in adult muscle utrophin expression is restricted to intramuscular nerves, blood vessels and myofibers, where it is mainly located in acetylcholine receptor rich crests at the neuromuscular junctions, and at the myotendinious junctions (Khurana 1991; Nguyen 1991; Ohlendieck 1991b). Here it recruits DGC proteins into a DGC-like complex and is probably involved in maintaining the structural integrity of the postsynaptic cytoskeleton (Love 1993; Matsumura 1992). In developing and regenerating skeletal muscle utrophin is located along the sarcolemma, where it is later replaced by dystrophin (Helliwell 1992; Lin 2000; Pons 1993).

In DMD patients and mdx mice utrophin expression is upregulated and the protein is located along the entire sarcolemma, where it probably compensates to some extent for

the lack of dystrophin (Galvagni 2002; Karpati 1993; Khurana 1991; Mizuno 1993; Mizuno 1994). This theory is supported by the finding that dystrophin-utrophin double knockout mice have a very severe phenotype (Deconinck 1997). It is obvious that the upregulated levels of utrophin in mdx mice and DMD patients cannot fully compensate for the loss of dystrophin. However, transgenic studies with mice carrying utrophin overexpressing cDNA constructs have shown that a 2-3 fold increase of utrophin expression in the muscle could improve the phenotype of mdx mice significantly, and that a 10-fold increase could prevent the dystrophic phenotype entirely (Tinsley 1998). Thus the upregulation of utrophin gene expression is a promising approach that may be therapeutically applicable to DMD patients. Drugs to enhance utrophin expression in cultured cells and animal models have been identified by Summit PLC (John Tinsley and Kay Davies, UK), BioMarin Pharmaceutical Inc. and PTC Therapeutics. Clinical trials are under preparation.

Myostatin inhibition

Myostatin inhibits muscle growth. Knock-out animals for myostatin leads to increased muscle formation in animals (Belgium blue cattle, Texel sheep, greyhounds, mice) and humans. This could compensate for the loss of muscle tissue in Duchenne patients and can be achieved by antibodies for myostatin. Myostatin antibodies have been tested in healthy volunteers and were deemed safe (http://www.clinicaltrials.gov/ct2/show/NCT00563810?term=myo-029&rank=1). They were consecutively tested in adult patients with muscle diseases (http://www.clinicaltrials.gov/ct2/show/NCT00104078?term=myo-029&rank=2). While treatment was safe, it did not result in an increase in muscle mass in the patients. However, patients were only treated for 28 days, which might not have been long enough.

Deacetylase Inhibitors

Pharmacological interventions that increase myofiber size counter the functional decline of dystrophic muscles as for myostatin inhibitors (Zammit 2002). Minetti and coworkers showed that deacetylase inhibitors increase the size of myofibers in dystrophin-deficient

(mdx) and a-sarcoglycan (a-SG)–deficient mice by inducing the expression of the myostatin antagonist follistatin3 in satellite cells. Deacetylase inhibitor treatment conferred on dystrophic muscles resistance to contraction-coupled degeneration and alleviated both morphological and functional consequences of the primary genetic defect (Minetti 2006). Colussi and colleagues elucidated the role of HDAC2 in the pathogenesis of Duchenne muscular dystrophy and indicate that HDAC2 inhibition by NO-dependent S-nitrosylation is important for the therapeutic response to NO donors in mdx mice for using deacetylase inhibitors in the pharmacological therapy of muscular dystrophies (Colussi 2008).

Idebenone

Buyse and coworkers tested SNT-MC17/idebenone in the mdx mouse, based on the drug's potential to improve mitochondrial respiratory chain function and reduce oxidative stress which is themajor cause of heart failure and death inDMD patients (Buyse 2009). In this study, 200 mg/kg bodyweight of either SNT-MC17/idebenone or placebo was given from age 4 weeks until 10 months in mdx and wild-type mice. Idebenone treatment significantly corrected cardiac diastolic dysfunction and prevented mortality from cardiac pump failure induced by dobutamine stress testing in vivo, significantly reduced cardiac inflammation and fibrosis, and significantly improved voluntary running performance in mdx mice. A Phase II extension trial where the effect of longer treatment was tested is now fully recruited and ongoing whilst a Phase III study has also been initiated and is currently recruiting patients.

PTC-124

PTC124 {3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid} is a chemical entity that selectively induces ribosomal readthrough of premature but not normal termination codons. Only patients with nonsense mutations would benefit from PTC124. However, 40% of the small mutations cause a frameshift (e.g., through a one or two nucleotide deletion or insertion, or by interfering with normal splicing), and here PTC would not be applicable (Aartsma-Rus 2009a). PTC124 activity, optimized using nonsense-containing reporters, promoted dystrophin production in primary muscle cells from humans and

mdx mice expressing dystrophin nonsense alleles, and rescued striated muscle function in mdx mice within 2–8 weeks of drug exposure. PTC124 was well tolerated in animals at plasma exposures substantially in excess of those required for nonsense suppression. The selectivity of PTC124 for premature termination codons, its well characterized activity profile, oral bioavailability and pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options (Welch 2007). PTC124 was safe in healthy volunteers. A phase II trial in Duchenne patients has been performed. Patients received different doses of PTC124 daily for 4 weeks. Treatment was tolerated well by patients and restored dystrophin clearly at highest doses. Trials to test whether this also results in functional improvement a longer clinical trail are currently ongoing in multiple centers in the USA and Europe.

Cell Therapy

In the last years, stem cells received much attention for their potential use in cell-based therapies for various human diseases. More than one clinical trial has been carried out with muscle stem cells in DMD patients (Mendell 1995; Torrente 2007). For several years after they were discovered, the satellite cell were considered as the only cells responsible for the growth and maintenance of skeletal muscle. With the improvements of cell-isolation technology, a number of markers were described to identify a lot of muscular and non-muscular subpopulations able to actively participate in myogenesis. Recent works described the partial identification and characterization of multi-lineage stem cells derived in culture from numerous adult tissues. Stem cell populations suitable for clinical experiments were found to derive from multiple region of the body at various stage of development such as satellite cells (Buckingham, 2006), muscle-derived stem cells (Qu-Petersen, 2002), side population (Asakura, 2002), bone marrow-derived stem cells (Dezawa et al., 2005), mesoangioblasts (Cossu, 2003), blood- (Gavina, 2006) and muscle-derived CD133+ stem cells (Benchaouir, 2007) and pericytes (Doherty, 1998). Montarras and colleagues (2005) were able to directly isolate a pure population of satellite cells from diaphragm muscle, unfortunately it appeared clearly that the growth of freshly isolated satellite cells in vitro significantly reduced their in vivo myogenic potential and that it would be very difficult to obtain a sufficient quantity of this kind of

cells. This is particularly important from a clinical standpoint since cell transplantation of autologous genetically corrected satellite cells to DMD patients is theoretically the ideal approach to minimize host immune rejection of donor cells (Price, 2007). The results obtained in the mouse model led to test the myoblast injection in DMD patients in phase I clinical trials. In these trials, donor satellite cells/myoblasts were isolated from muscle biopsies from first-degree relatives of the affected children and were grown in culture (Daston, 1996; Seale, 2004). These trials demonstrated that myoblast transplantation is an inefficient technique; the efficiency of the dystrophin production in muscle fibres of DMD patients was very low (~1%) and there was no functional or clinical improvement in the children (Péault, 2007). Recent studies suggested the existence of a population of a multipotent muscle-derived stem cell (MDSC) that resides within skeletal muscle sharing the ability to self renew and to differentiate into other mesodermal cell types (Sarig, 2006; Tamaki, 2007). Although MDSCs were amenable to therapeutic applications because they are easy to proliferate in vitro and able to migrate through the vasculature, and also multipotent (Deasy 2001 and 2005), further studies to elucidate the physiological location of MDSCs are required prior to the clinical use of these cells.

In the last decade, various groups isolated and characterised the contribution of various nonmyogenic cells in the regeneration of skeletal muscle, such as bone marrow-derived cells (BMDC) and the circulating haematopoietic cells (Lennon, 2006). Adult bone marrow of many species contains a rare population able to extrude the vital dye Hoechst 33342 called Side Population (SP) (Goodell, 1997). Asakura et al., demonstrated that when injected into regenerating mouse mSP can differentiate into functional satellite cells expressing Myf5, Pax7 and desmin (Asakura, 2002). In contrast to satellite cells or myoblasts, mSP cells are able to migrate from the blood stream into muscle, a desirable feature for widespread distribution of a therapeutic cell type.

Mesoangioblasts are multipotent progenitors of mesodermal tissues, physically associated with the embryonic dorsal aorta in avian and mammalian species. Early studies of Cossu and Bianco (2003) concerning the capacity of mesoangioblasts to differentiate in various mesodermal phenotypes qualified these progenitors as a novel class of stem cells. Cossu and coworkers transduced mesoangioblasts with a lentiviral vector expressing human microdystrophin and injected scid/mdx mice and dystrophic GRMD dogs, treated with a combination of immunosuppressive drugs and steroids (Sampaolesi 2006; Cossu 2007). They showed that modified mesoangioblasts

produced dystrophin positive myofibres in these animal models (Sampaolesi 2006; Cossu 2007). Nevertheless there was not a close relationship between the number of dystrophin-positive myofibres and the amelioration observed (Davies, 2006) and it was also observed that the improved muscle health determined by reduced serum kinase levels could be an effect of the immunosuppressive drugs and also that isolation from muscle blood did not exclude the presence of other muscle stem cells (Davies, 2006; Radley, 2006).

Pericytes wrap around the vascular tube and interdigitate with the endothelial cells in the basement membrane of the vessels, playing a fundamental role in the maintenance of microcirculation functionality. Pericytes can be mobilized from adult bone marrow under ischemic conditions and utilized for their contractile capabilities and their multiple cytoplasmic processes. Dellavalle and coworkers demonstrated that pericytes had an high capacity of myogenic differentiation because they gave rise to a high number of muscular fibres when injected into scid/mdx mice (Dellavalle, 2007). These stem cells could represent a good candidate for muscle therapy because they could be isolated from a muscle biopsy and so easily accessible, they can be cultured in vitro without loss of stem-cell properties and are able to regenerate skeletal muscle after muscular and arterial injection (Morgan, 2007). Nevertheless, it would be important to determine, firstly, whether transplanted pericytes can fully reconstitute the satellite cell niche as a real functional stem cell (Morgan, 2007).

CD133+ cells are considered to be haematopoietic and endothelial stem cells of bone marrow origin that could give rise to both endothelial cells and myoblasts (Péault, 2007). It has been demonstrated the stemness of circulating human CD133+ cells and their ability to restore dystrophin expression and eventually regenerate the satellite cells pool in dystrophic scid/mdx mouse after intra-muscular and intra-arterial delivery (Gavina, 2006). Two different CD133+ subpopulations were studied: blood and muscle derived CD133+ cells; muscle CD133+ cells showed a better muscle regeneration in term of spreading and number of positive fibres in comparison with the results obtain with blood-derived stem cells (Benchaouir, 2007). Several things need to be ameliorated such as the potential to enhance proliferation of CD133+ cells in culture and storage for repeated treatments, the relative efficiency of blood-compared with muscle-derived cells to contribute muscle nuclei, a strategy to deliver myogenic cells chronically to the various sites of sporadic regeneration that occur in muscular dystrophies.

Gene Replacement

rAAV vectors are derived from non-pathogenic and non-replicative parvoviruses (Athanasopoulos 2004) with a single-stranded DNA genome of approximately 4.7 kb (Laughlin 1983; Samulski 1982; Hermonat 1984; Srivastava 1983; Tratschin 1984) and a 20 nm regular icosahedral viral particle (Xie 2002). As a vector for gene transfer, all AAV genes (Rep and Cap) have been eliminated, with the exception of the 145 bp inverted Gene Therapy terminal repeats (ITRs) flanking the foreign gene(s) such as therapeutic or reporter genes. rAAV vector genome, lacking the Rep 78 sequence, primarily persists in an extrachromosomal form (Song 2004) – known as episomal chromatin – in the nucleus of non-dividing cells such as muscle (Penaud-Budloo 2008). This unique viral vector has the distinct advantage of being capable of infecting a wide range of host cell types including dividing and non-dividing cells (Xiao 1996; Kessler 1996). This property makes rAAV a particularly good vector for treating diseased tissues such as dystrophic muscle since muscle cells do not undergo rapid cellular proliferation and division. The potential benefits of utilizing rAAV for the treatment of muscle disorders include superior long-term gene transfer efficiency, less immune response to the virus and a lack of toxicity in muscle, liver and heart (Manno 2003; Jung 2001; Gray 2008), all of which are often associated with the use of other viral vectors. Two major disadvantages of rAAV vectors include the lack of muscle-specific gene transfer and the limited packaging capacity of the transgenes (< 5 kb), which affects the selective gene transfer capability of rAAV vector and also limits the choice of the genes of interest. Certain AAV serotypes demonstrate natural tropisms for muscle, making them potential candidates for muscle-directed gene transfer for the treatment of muscle disorders (Athanasopoulos 2004; Wang 2000; Gregorevic 2006; Wang 2009; Inagaki 2006). rAAV1 and rAAV2 are commonly applied for direct delivery and local treatment, while rAAV6, rAAV8 and rAAV9 are usually utilized for systemic delivery as they are the most efficient serotypes for crossing the blood vessel barrier to attain systemic gene transfer for whole-body muscle delivery, including both skeletal and cardiac muscles. rAAV capsid is an essential component of this vector, as it is involved in cell binding, internalization and trafficking within the targeted cells. To perform high-efficiency transduction at lower doses, rAAV capsid mutants have been developed to improve receptor binding and virus uptake by altering capsid plasticity (Schaffer 2004) and by the inclusion of a muscle-targeting peptide displayed on AAV2, which has been found to

improve muscle tropism during systemic delivery (Yu 2009). Of the currently used rAAV vectors, rAAV2 has been commonly used in human clinical trials (Rodino-Klapac 2007) because it has been studied extensively and is not associated with any known diseases. The low efficiency of rAAV2 transduction, however, limits its application to muscle gene delivery when compared with other serotypes. In addition, since AAV2 virus arises from humans, there are a considerable number of people with preexisting neutralizing antibodies (Chirmule 2000; Sun 2003; Wu 2006) which could affect the outcome of gene therapy clinical trials for the treatment of muscle diseases. More recently, there has been an effort to generate low-immunogenic, cell- and tissue type-specific rAAV vectors in an attempt to minimize human host immune response. An example of this is rAAV2.5, which has been used for the first gene therapy clinical trial for the treatment of Duchenne muscular dystrophy (DMD) (http://www. clinicaltrials.gov/ct2/show/NCT00428935?term=NCT00428935&rank=1). This chimeric viral vector was created by Dr Samulski's group (The University of North Carolina at Chapel Hill, US) through the alignment of both AAV1 and AAV2 capsid sequenze and resulted in 40-fold increase transduction efficiency. rAAV-based gene therapy represents one of the most promising approaches to aid in the treatment of DMD which has been demonstrated in various animal models of DMD including dystrophin deficient (mdx) mice (Wang 2000; Watchko 2002), dystrophin/utrophin double-knockout (dys-/-:utro-/-) (dKO) mice (Gregorevic 2006; Wang 2009) and canine models (Wang 2007). However, the local treatment of skeletal and cardiac muscles for the treatment of muscular dystrophies is limited (Watchko 2002; Monahan 1998; Li 1999; Xiao 2000; Melo 2002; Yue 2003; Chu 2004; Kaspar 2005) because the muscular dystrophies afflict most all of the muscles of the body and the delivery of the transgene needs to be systemic. Intraperitoneal (i.p.) (Wang 2009) and intravenous (i.v.) (Wang 2005; Zhu 2005) injections are often used for the treatment of experimental animal models of DMD and limb-girdle muscular dystrophies (LGMD); however, some serotypes of rAAV, like most other viral and non-viral vectors, are inefficient at penetrating the blood vessel–tissue barrier when administered via the blood circulation. To achieve rAAV vector systemic delivery, both physical and pharmaceutical methods are being developed to surmount this barrier (Grrelish 1999; Gregorevic 2004), but all the methods currently under investigation are associated with severely invasive procedures and/or pharmaceutical side effects. Hence, for systemic gene delivery, it is important to identify

an appropriate rAAV serotype for skeletal and cardiac muscle transduction in order to efficiently deliver transgenes systemically to treat genetic diseases of muscle. Although AAV serotypes 6 and 8 have been commonly used for systemic delivery to skeletal and cardiac muscles (Gregorevic 2006; Wang 2005; Zhu 2005), a newly discovered AAV vector, serotype 9, has shown superior skeletal and cardiac gene transfer capacity (Inagaki 2006; Pacak 2006) when it is delivered via a single i.v. injection to neonatal normal dogs (Yue 2008) and young adult GRMD dogs (Kornegay 2009; Li 2009). The 5 kb packaging limitation of rAAV vector limits its utility for delivery of the dystrophin gene (11 kb cDNA) (Wang 2000), therefore we have created a series of novel minidystrophin genes (< 4.2 kb) that are readily packaged into AAV vectors (Wang 2000; Wang 2009; Watchko 2002; Wang ET 2008). In others studies (Wang 2000; Gregorevic 2006; Sakamoto 2002), the rod structure and length of the mini-dystrophin protein is crucial for dystrophin's proper function, evidenced by the fact that at least one mini-dystrophin construct needs four rods and two hinges to have the capability to protect skeletal muscle fibers from the dystrophic processes. In addition to the achievements being made in minidystrophin replacement therapy in small and large animal models, important progress has been made in other areas such as exon-skipping strategies which is currently generating very exciting results (Aartsma-Rus 2009a; Arnett 2009; Goyenvalle 2004; Lu 2005; Nakamura 2009; Ruszczak 2009; Trollet 2009; van Ommen 2008). To overcome the limitation of low potency and inefficiency of antisense oligonucleotide (AONs)- mediated exon skipping in systemic delivery, rAAV vectors have been developed to express an antisense sequence linked to a modified U7 (Goyenvalle 2004) or U1 (Denti 2006) small nuclear RNA. As a result, exon skipping could remove the exons containing nonsense mutations and restore the normal reading frame of the dystrophin gene (Goyenvalle 2004; Denti 2008). Importantly, systemic delivery of AONs–AAV constructs through tail vein injections in mdx mice has resulted in the restoration of functional dystrophin throughout the musculature of the body and the improvement of muscle strength. A first clinical trial where patients received local AAV-microdystrophin injections in the arm muscle was performed in the USA (Mendell, Xiao Xiao and Samulski). Results of this trial are pending.

Splicing Modulation

Splicing mechanism

In most eukaryotic genes the coding sequence is dispersed over several small exons that are interrupted by non-coding introns. The average intron size is 3 kb, but much longer introns have been reported, for instance most of the introns of the DMD gene (Ahn 1993; Goldstrohm 2001). Introns are removed from the pre-mRNA and the exons joined to each other in a process called pre-mRNA splicing (see Figure 7).
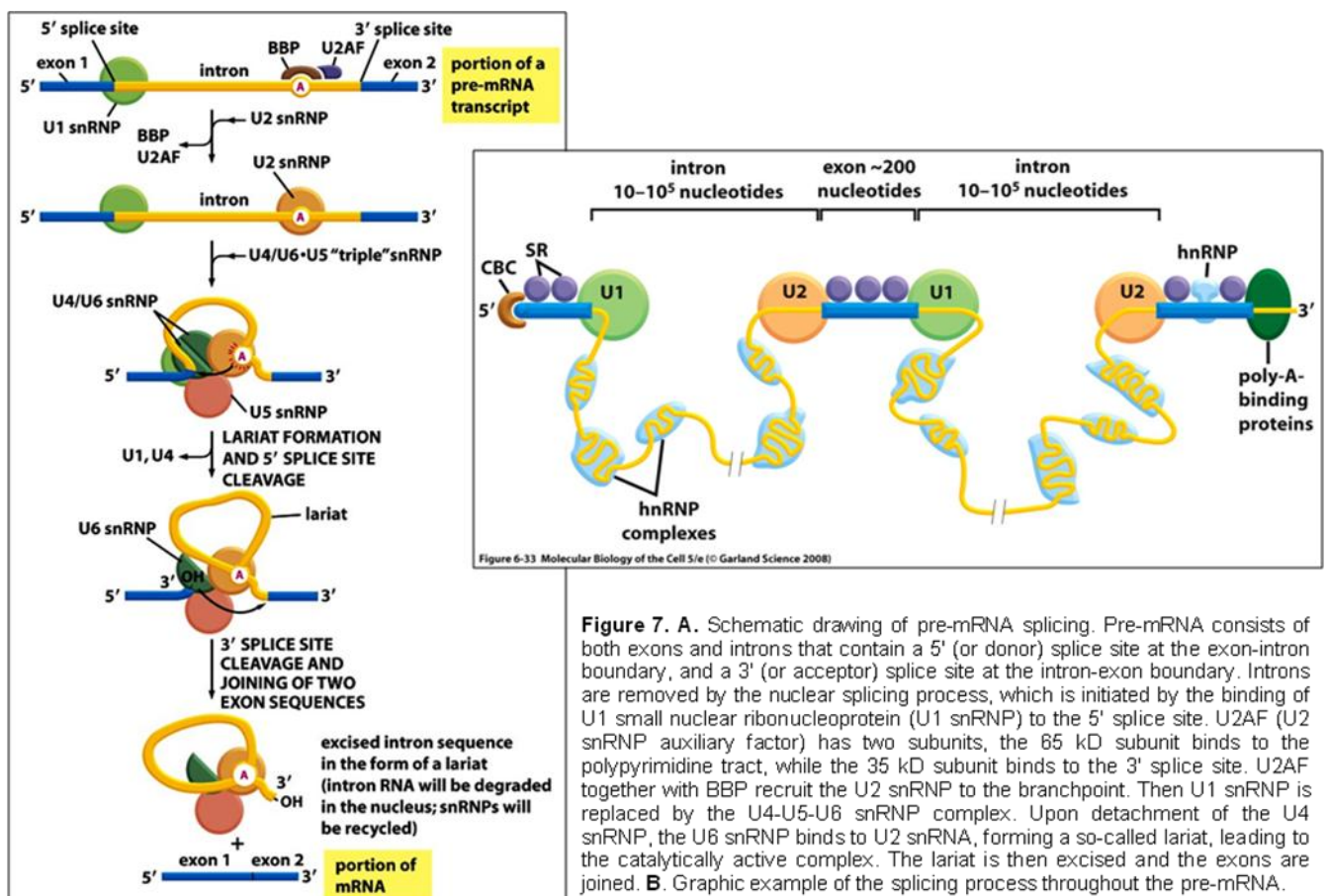

Figure 7. A. Schematic drawing of pre-mRNA splicing. Pre-mRNA consists of both exons and introns that contain a 5' (or donor) splice site at the exon-intron boundary, and a 3' (or acceptor) splice site at the intron-exon boundary. Introns are removed by the nuclear splicing process, which is initiated by the binding of U1 small nuclear ribonucleoprotein (U1 snRNP) to the 5' splice site. U2AF (U2 snRNP auxiliary factor) has two subunits, the 65 kD subunit binds to the polypyrimidine tract, while the 35 kD subunit binds to the 3' splice site. U2AF together with BBP recruit the U2 snRNP to the branchpoint. Then U1 snRNP is replaced by the U4-U5-U6 snRNP complex. Upon detachment of the U4 snRNP, the U6 snRNP binds to U2 snRNA, forming a so-called lariat, leading to the catalytically active complex. The lariat is then excised and the exons are joined. **B**. Graphic example of the splicing process throughout the pre-mRNA.

This process is coordinated by a catalytical complex called the spliceosome, which consists of 5 small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6 snRNP), and further involves over 60 general splicing factors (Will 2001). Each snRNP contains an RNA part, complementary to its target sequence in the premRNA, and a protein part, to bind to other snRNPs or splicing factors. The initial step in the process of splicing is the binding of U1 snRNP to the 5' splice site. Then, the U2 snRNP is recruited to the branchpoint sequence by the 65 k subunit of the U2 auxiliary factor (U2AF), that binds

to the region of the 3' splice site. The U4-U5-U6 tri snRNP complex then replaces U1 snRNP at the 5' splice site. When U4 snRNP detaches from the complex, U6 snRNP is free to bind to U2 snRNP, leading to a mature catalytically active spliceosome. The lariat, containing the intron and snRNPs, is then excised and the exons are joined together to form a mature mRNA transcript (Stojdl 1999). Correct splicing requires the accurate identification of the branchpoint, the 3' (acceptor) and the 5' (donor) splice sites of exons by the spliceosome. However, in higher organisms the consensus sequences for these sites is weakly defined (Zhang 1998). The only nucleotides that are invariably found in every intron are the GT at the start, the AG at the end and the A at the branchpoint (except for a small subclass of AT-AC introns (Tarn 1996) and GC-AG introns (Senapathy 1990)). In general 5' splice sites are determined by the sequence AG│**GT**RAGt (where bold letters are invariably found, capitalized letters occur in over 50% of analyzed splice sites and non-capitalized letters were found in over 33%, R is a purine (G or A) and │ indicates the exon-intron boundary) (Zhang 1998). 3' Splice sites contain an intronic polypyrimidine stretch (pyrimidine is C or T – indicated by the symbol Y), which can vary in length between 2 and 32 nucleotides. The 3' splice site consensus sequence is $(Y)_{2-32}NC\mathbf{AG}│GN$ (where │ is the intron-exon boundary) (Zhang 1998). The branchpoint is even more ambiguous: NNCTV(A)Y (where V is A, C or G and (A) is the branchpoint) (Zhang 1998). Online programs containing information of numerous human splice sites can be used to predict the likelihood that a given sequence contains 5' or 3' splice sites (e.g. Pertea 2001: www.tigr.org/tdb/GeneSplicer/gene_spl.html ; Reese 1997: www.cse.ucsc.edu/~dkulp/cgi-bin/genie). Potential splice sites have a value between 0 and 1, where 1 is a site that matches the consensus perfectly (a strong splice site) and 0 denotes the absence of an identifiable potential splice site. However, cryptic (i.e. non genuine) splice sites with higher predicted values are present throughout the introns, sometimes located near weaker genuine splice sites that are nevertheless recognized by the spliceosome. Thus, the splicing machinery can distinguish between real and cryptic splice sites. This is mediated by the binding of spliceosomal components to genuine 3' and 5' splice sites. Many (if not all) exons contain exonic splicing enhancer (ESE) sequences (reviewed in (Cartegni 2002)). A subgroup of splicing factors, called the SR proteins, can bind to these ESEs. SR proteins (reviewed in (Stojdl 1999)) are localized in the nuclear speckles. They contain one or two RNA recognition motive sequences (RRM) and a C-terminal serine-arginine

stretch (RS domain), involved in protein-protein interactions with other splicing factors, such as the RS domain present in U1 and U2AF. Upon binding of an SR protein to an ESE, the SR protein recruits U1 snRNP and U2AF to their respective sites to aid splicing of an (weakly defined) exon. Different SR proteins have been described (SF2/ASF, SC35, 9G8, SRp75, SRp55, SRp40, SRp20, SRp30c, p54 and Tra2β), which have redundant functions. Initially discovered ESEs were invariably purine rich. This is probably due to the fact that these sequences can be bound by SF2/ASF and SC35, the most abundant SR proteins (Tacke 1995). However, recently AC-rich ESEs have also been described, and it appears that SR proteins are able to recognize a variety of sequences (Cartegni 2002; Coulter 1997). Since exons contain the coding sequence, which does not allow for much flexibility, the lack of a well-defined consensus is not surprising. It does imply, however, that it is difficult to predict which regions in an exon contain ESE sequences. The binding sites of SF2/ASF, SC35, SRp40 and SRp55 have been analyzed in detail and these results are implemented in ESEfinder, which is a websource that predicts ESE sites (Cartegni 2003) (see below). The observation that a mutation in a 5' splice site often also inactivates the upstream 3' splice site and thus results in the skipping of the exon, led to the exon definition model (Robberson 1990): the exon is the first unit to be recognized during early spliceosome formation, and is defined by the binding of splicing factors first to the 5' and then to the 3' splice sites. Subsequently the intervening intron is excised. It has been shown that SR proteins can form a bridge across the exon to help exon definition (Achsel 1996). In addition to constitutive splicing, 92-94% of human genes undergo alternatively splicing (Wang ET 2008), meaning that different splice sites or even exons are used in different transcripts of the same gene (see Figure 8). Exons that are alternatively spliced are characterized by weak splice sites and often require binding of (tissue specific) splicing factors for proper recognition by the spliceosome. In addition to ESEs, alternatively used spliced exons usually contain intronic or exonic splicing silencers. These can be bound by members of the heterogeneous nuclear ribonucleoprotein family (hnRNPs), which are the inhibitory counterparts of the SR proteins (Chen 1999). Both hnRNPs and SR proteins are differentially expressed during different developmental stages and in different tissues. The competitive binding of inhibitory and enhancer factors ultimately determines whether a certain splice site is used or not (Cartegni 2002).
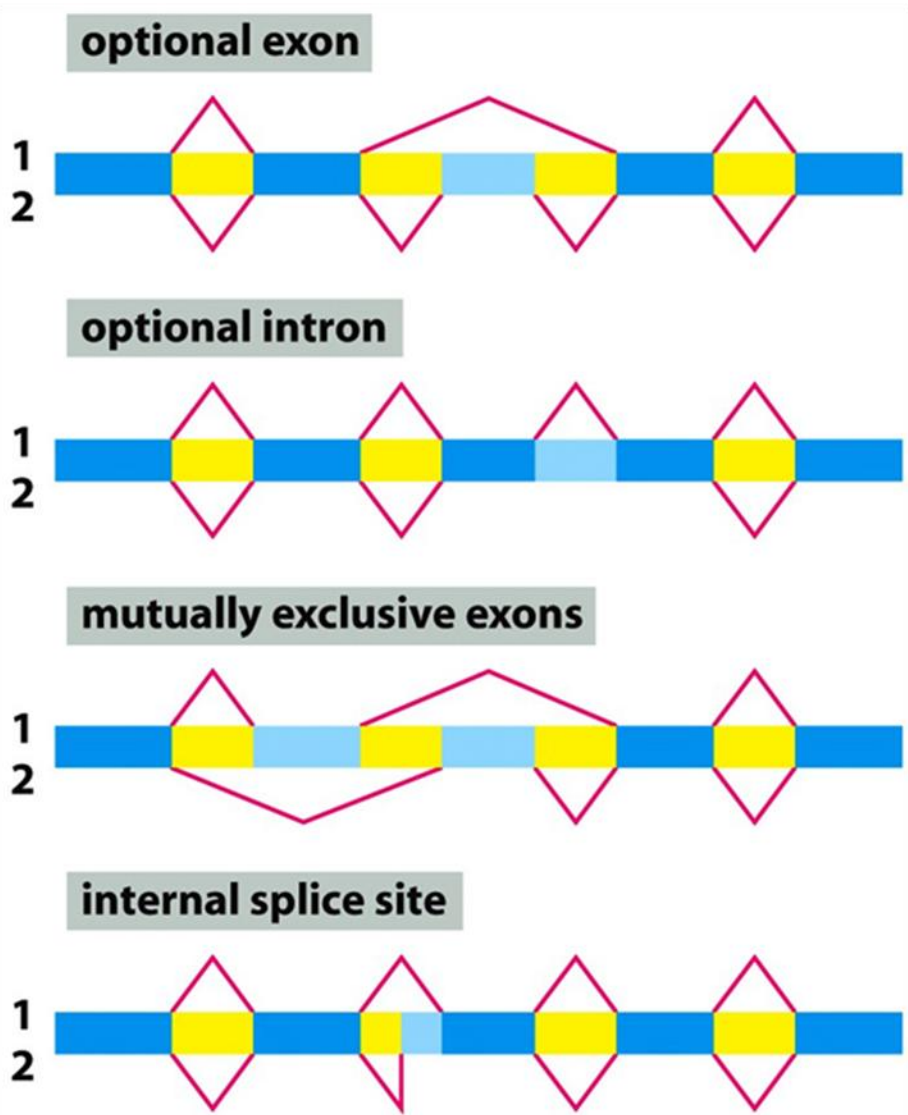
Figure 8. Schematic depiction of the different modes of alternative splicing. Alternatively spliced exons (exon is either skipped or included), mutually exclusively spliced exons and internal splice sites are shown. Other alternative splicing events are possible as alternative 5' splice sites, alternative 3' splice sites, intron retention (i.e. the intron is either included or spliced out).

Antisense-mediated restoration of normal splicing – Rational for exon skipping

The group of Kole was first to apply AONs to modulate the splicing (Dominski 1993). They targeted AONs to cryptic 3' and 5' splice sites in the β-globin gene, which are associated with β-thalassemia. AON treatment restored normal splicing for several different mutations in an in vitro splicing assay.

The observation that out-of-frame mutations in the DMD gene generally result in a severe phenotype, whereas in-frame mutations result in a milder phenotype prompted investigators to try and restore the reading frame (Monaco 1988)(Figure 9). For instance, a deletion of exons 48-50 creates a frame-shift and a premature stop codon in exon 51, resulting in a truncated protein and DMD. A deletion of exon from 48 to 51, on the other hand, is in-frame, can generate an internally deleted dystrophin and is found in

mildly affected BMD patients (Aartsma-Rus 2009a). Thus inducing the skipping of exon 51 in the pre-mRNA of DMD patients with an exons 48-50 deletion, would restore the reading frame, allow the generation of an internally truncated dystrophin and convert a severe DMD into a milder BMD phenotype.
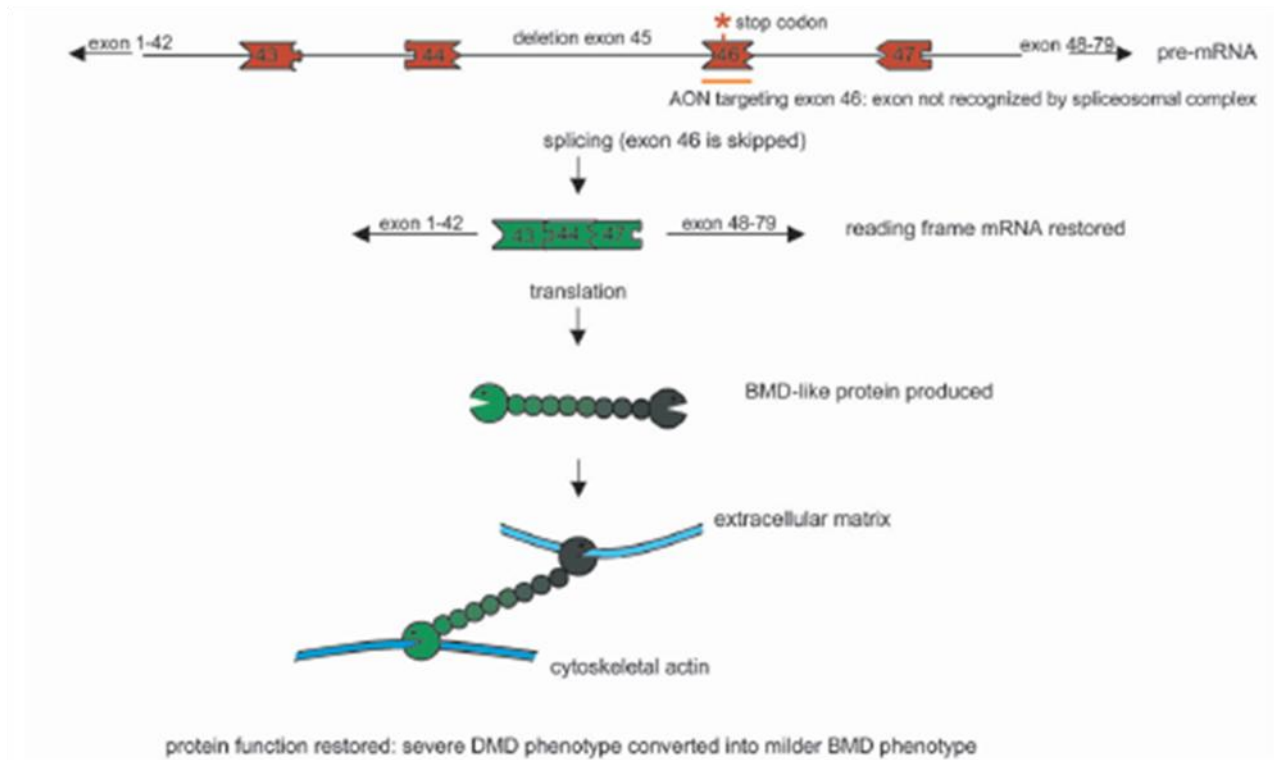

Figure 9. Schematic depiction of the reading frame restoration approach. In this example a DMD patient suffers from a deletion of exon 45, which results in a premature stop codon in exon 46. Upon binding of an antisense oligoribonucleotide (AON) specific for exon 46 (indicated by an orange line), this exon will be "skipped" by the spliceosomal machinery and exon 44 will be joined to exon 47, resulting in an in-frame mRNA, which can be translated into a semi-functional BMD-like dystrophin. In this way the severe DMD phenotype is converted into a milder BMD phenotype (Picture kindly provided by dr. Annemieke Aartsma-Rus).

For most DMD patients one or more exons need to be skipped in order to restore the open reading frame (Tuffery-Giraud 2009; Aartsma-Rus 2009a; Flanigan 2009). Exon skipping can be induced by AONs targeting the splice sites, the branchpoint or exon-internal sequences (Aartsma-Rus 2007). In each case, AON binding will hide the exon from the splicing machinery and consequently the targeted exon is skipped from the mRNA. This allows the production of internally deleted, partially functional dystrophin proteins, as found in BMD patients and thus has the potential to convert the severe DMD into a milder BMD phenotype.

However some exceptions to the rule do exist for example in BMD patients (out-of-frame BMD). This is partly explained by the high incidence of 5' gene deletions in BMD patients (a region known to be a hotspot for exceptions), and due to complex splicing patterns in which a subset of transcripts show deletions larger than gDNA (exon-skipping)(Kesari 2008). Another point to consider is that not all the in-frame dystrophin proteins are functional; although the dystrophin protein primarily consists of (partially) redundant domains, it needs its actin- and β-dystroglycan-binding domains to link the cytoskeleton to the extracellular matrix. Mutations affecting the domain that binds to β-dystroglycan (exon 64-70) render the protein non functional regardless of whether mutations disrupt the reading frame or not (Aartsma-Rus 2006a). The link to actin is achieved through 3 actin-binding domains encoded by exon 2-8 (2 domains) and 35-44. Dystrophins lacking all three domains are non functional, while mutations where at least one binding is maintained generally lead to BMD, although patients lacking the first 2 actin-binding domains often have a more severe phenotype than "typical" BMD patients carrying an in-frame deletion in the hotspot region (Aartsma-Rus 2006a).

Antisense Oligonucleotides (AONs) design

More than one study has tried to point out rules to use when designing AONs to induce effective exons kipping throughout the dystrophin gene (Errington 2003; Aartsma-Rus 2005; Wilton 2007; Mitrpant 2009a; Popplewell 2009). In the first report by Errington and colleagues the efficiencies of exon 19 specific AONs, targeting either the 5' splice site or the ESE site deleted in DMD Kobe, were compared in human and mouse myotube cultures (Errington 2003). RT-PCR analysis revealed that both types of AONs could induce exon 19 skipping, but the exon internal AONs were somewhat more efficient. This rule could not be extended to all the exons as we learnedfrom the work by Wilton and co-workers (Wilton 2007). In 2005 Aartsma-Rus and colleagues retrospectively analyzed 114 exon-internal AONs (2'-O-Methyl phosphorothioate modified AONs - 2OMePS) used to skip 36 out of 78 skippable exons identifying 4 parameters which were significantly different between effective and non effective AONs: effective AONs showed higher values of SR proteins SF2/ASF, SC35, SRp40 and a smaller absolute distance from 3' splice site (Aartsma-Rus 2005). Wilton and colleagues were able to skip almost all 78 exons with different efficiencies allowing a differentiation of the exons

in 4 groups: Types 1, 2, and 3 exons refer to exons that can be removed with high (greater than 30% relative to the intact transcript), medium (between 10 and 30%), and low (less than 10%) levels of exon-skipping efficiency, respectively. Type 4 exons include those from which additional flanking exons are also removed and/or more than one AON was required for efficient exon skipping. The only rule the authors could infer from this huge amount of experiments was that AONs targeting the first half of the exon were more effective that AONs targeting the second half of the exons (Wilton 2007). The same group in 2009 in an article entitled "Rational Design of Antisense Oligomers to Induce Dystrophin Exon Skipping" concluded: "we report substantially different levels of exon skipping induced in normal and dystrophic human myogenic cell lines and propose that animal models or artificial assay systems useful in initial studies may be of limited relevance in designing the most efficient compounds to induce targeted skipping of human dystrophin exons for therapeutic outcomes" (Mitrpant 2009a). This led to further studies by Popplewell and colleagues who analyzed 66 AONs (morpholinos – PMOs) targeting 5 exons: exons 44, 45, 46, 52 and 53. The authors concluded that active PMOs were longer, bind better to their target site (lower free energy compared to non effective AONs), target sites close to the acceptor splice site, overlapped with open conformations areas (as defined by hybridization or RNA secondary structure prediction software) and interfere with SR protein binding (Popplewell 2009). Bioinformatic tools helped in understanding which parameters should be considered when analyzing AONs (Wee 2007) although real rules are still missing.

AON modifications

As AON-mediated inhibition of gene expression and modulation of splicing gained more attention, modifications of the phosphate backbone and the sugar moiety to improve the biological, biochemical and biophysical characteristics of AONs were developed and evaluated. As a result a wide variation of different AON analogues is now available (reviewed in (Kurreck 2003)). The earliest modification involved the change of one of the non-bridged oxygen atoms in the phosphate group into a sulphate atom (De Clercq 1969). This resulted in phosphorothioate DNA (PS), which had an increased half-time when compared to normal DNA. However, PS oligos induce RNase H degradation when bound to their RNA target, which is pivotal in sequence specific knockdown of

genes, but detrimental in AONmediated modulation of pre-mRNA splicing. Modifications at the 2'-O position of the ribose of resulted in RNA oligos that had an increased affinity for RNA and did not induce RNase H (Dominski 1993; Sproat 1989). The most frequently used 2'-O modifications are 2'-O methyl (2OMe) and 2'-O-methoxyethyl (2OMOE), where the 2'-O of the ribose are substituted by a methyl (CH_3) group and a methoxyethyl ($C_2H_4OCH_3$) group, respectively (Kurreck 2003). However, PS modifications are still required as AONs with 2'-O modifications are not very resistant to nucleases. Using these analogues efficient exon skipping or redirection of aberrant splicing can be achieved at 100 nM in human cells and at even lower concentration in murine cells (Aartsma-Rus 2003; Bremmer-Bout 2004; Errington 2003; Mann 2002).

Peptide nucleic acids (PNA) are only remotely related to DNA oligos (reviewed in (Larsen 1999)). In PNAs the phosphate backbone is replaced by an achiral, uncharged, relatively flexible backbone consisting of 2-aminoethyl glycine units. The nucleobases are attached to these units by methylenecarbonyl linkers. PNAs are resistant to biological degradation by nucleases, proteinases and peptidases, and can bind to DNA and RNA with high affinity and specificity. The main problems with PNAs arise from their hydrophobic nature, which makes them insoluble and difficult to transfect (Braasch 2002a). However, the peptide backbone is ideally suited for the attachment of carrier groups, to increase the cellular uptake. In addition, it has been shown feasible to increase the solubility and free cellular uptake to a great extent by adding 4 basic lysine residues to the PNA (Sazani 2001 and 2002). Yin and colleagues achieved exon skipping using peptide conjugated PNA AONs (Yin 2008a and 2010) although it has been reported that PNAs are rapidly cleared in vivo, making them less attractive as therapeutic compounds (Mardirossian 1997).

Morpholinos contain a morpholino moiety instead of the ribose sugar and phosphoroamidate intersubunit linkages instead of phosphodiester bonds (Kurreck 2003). They have an affinity that is comparable to DNA oligos, and are completely nuclease resistant and non-toxic (Summerton 1999). Due to the neutral nature of the morpholino backbone, undesired binding of proteins is unlikely (Summerton 1999). However, an unbeneficial consequence is that the cellular uptake is limited and nuclear uptake is even more problematic (Summerton 1999). The limited nuclear uptake is advantageous for translation inhibition studies, since translation takes place in the cytoplasm, and may explain why morpholinos have been found to be potent inhibitors of

protein expression in embryonic zebra fish (Nasevicius 2000). Modulation of pre-mRNA splicing, on the other hand, occurs in the nucleus. Low levels of exon 46 skipping were achieved relatively using 1000 μM of a morpholino annealed to a sense DNA leash, with ethoxylated PEI (EPEI) as a transfection reagent (Aartsma-Rus 2004b). Since the authors observed both nuclear and cytoplasmic staining of the fluorescent morpholino, they concluded that the low efficiency of morpholinos might be partly due to limited nuclear uptake. The group of Wilton has recently showed improved nuclear uptake by annealing the morpholino to a partially overlapping sense DNA leash, delivered as a cationic lipoplex (Gebski 2003). Upon entering the nucleus the leash is removed by nuclease degradation (starting at the non-annealed non-overlapping base pairs), freeing the morpholino to bind to the target sequence. Using an optimised leash, high levels of exon 23 could be induced in mdx myotube cultures at 300 nM, and significant levels were still detectable at 5 nM concentrations.

Locked nucleic acids (LNA) are locked in the 3' endo-conformation by a methylene bridge that connects the 2'-O to the 4'-C of the ribose (Obika 1998). As a consequence LNAs are inflexible and have a very high affinity for RNA and DNA. In addition LNAs are non-toxic and nuclease resistant (Wahlestedt 2000). Unfortunately, the extreme high affinity of the LNAs may be a disadvantage rather than an advantage. LNAs longer than 15 nucleotides will efficiently self-anneal and LNAs are not very sequence specific (Braasch 2002b; Fluiter 2003, Aartsma-Rus 2004b).

Alternatively, chimeras containing a limited amount of LNA or ENA (Ethylene bridged nucleic acids) nucleotides have been used (Arzumanov 2001; Takeshima 2003; Yagi 2004). For these chimeras the affinity for the target RNA is increased when compared to 2OMePS AONs, but not so high as full-length LNAs and ENAs, meaning that they are more sequence specific. An RNAENA chimera targeting exon 41 of the dystrophin gene has successfully been used to induce exon 41 skipping in patient derived myotubes and dystrophin expression was observed in 80% of treated myotubes (Takeshima 2003). This chimera is currently being tested in a phase I/II clinical trial in Japan (Matsuo 2004).

Multi exon skipping

Multiexon skipping (simultaneously skipping a stretch of exons) has been proposed as a method to address larger groups of mutations with a similar treatment combination (Aartsma-Rus 2004a; Tuffery-Giraud 2009). There are a number of large deletions that would be suitable, since they would apply to a large group of patients and/or have been found in very mild BMD patients, suggesting that the resulting dystrophin is largely functional (Beroud 2007). Unfortunately, while multiexon skipping of a small number of exons is feasible (Aartsma-Rus 2004a; Fall 2006; McClorey 2006a; Yokota 2009), multiexon skipping of larger stretches has so far not been successful (Aartsma-Rus 2006b; van Vliet 2008).

Single exon skipping

In vitro experiments

Single Exon skipping could restore the open reading frame, avoiding premature termination codons, in 64% of all DMD causing mutations (Aartsma-Rus 2009a). AON-mediated exon skipping feasibility in the DMD gene has been tested and achieved using several AON chemistries: the first attempts were performed phosphorotioate backcone AONs (PS AON) which can however activate RNase H. To optimize the AONs chemistry and sequences most of the experiments wew perdormed on mdx mouse cells using 2'-O-methyl modified AONs (2OMe-RNA)(Dunckley 1998; Wilton 1999; Mann 2001), 2'-O-methyl phosphorothioate modified AONs (2OMePS) (Mann 2002; Errington 2003; Fall 2006; Adams 2007; Mitrpant 2009a), morpholinos (PMOs) (Gebski 2003; Fall 2006), peptide conjugated morpholinos (Moulton 2007 on mdx cardiomyocytes), PNAs and peptide conjugated PNAs (Yin 2008a). Several experiments were also performed on human myoblasts using 2OMe-RNA (Kinali 2009), 2OMePS AONs (van Deutekom 2001; Aartsma-Rus 2002; Aartsma-Rus 2003; Errington 2003; Aartsma-Rus 2004b; van Deutekom 2007; Wilton 2007; Adams 2007; Mitrpant 2009a; Spitali 2009), PMOs (Adams 2007; Popplewell 2010; Aartsma-Rus 2004a), U7 constructs (Goyenvalle 2007), PNAs and LNAs (Aartsma-Rus 2004b). Exon skipping has also been assessed

on human muscle explants (McClorey 2006b), 4^{cv} mice derived cells (Mitrpant 2009b) and CKCS-MD dog derived myogenic cells (Walmsley 2010).

In **paper 1** we achieved single exon skipping for small mutations. Single exon skipping can be applied for small mutations lying within in-frame exons only, 11% of all DMD patients are eligible for such a treatment (Aartsma-Rus 2009a). Therefore, the general aim of this study was to confirm the applicability of single exon skipping to small mutations. We also intended to evaluate whether "universal" or "private," mutation-specific AONs were more effective in inducing the exon skipping of mutated dystrophin exons, effectively impacting the design of future therapies for these patients. It is, in fact, known that small mutations can disrupt exon splicing enhancers (ESEs) and silencers (ESSs), splicing regulatory sequences devoted to the exon recognition process (Cartegni 2003). Mutations located in ESE and ESS elements may affect splicing. Consequently, a significant proportion of exonic point mutations could exert an effect completely different as would be predicted from the genetic code. Positive and negative regulatory elements play opposite and fundamental roles in a finely tuned exon recognition process (Nielsen 2007), which need to be taken into account when interfering with the splicing process. Because mutations lying within ESEs may strongly affect the splicing process (Solis 2008), we used this double approach to point out this relevant topic. We have identified 54 unrelated DMD patients carrying small mutations in the DMD gene, of which 32 are novel. Among 24 patients carrying a mutation in an in-frame exon, we selected 5 mutations in 5 different exons to be modulated (exons 10, 16, 26, 33 and 34). For the five selected dystrophin exons, the splicing specific exon skipping was effectively reproduced in four cell free splicing assays and in all five myogenic cells out of five selected mutated exons. When comparing the two different assays it was demonstrated that, in the absence of patient-derived cells availability, the cell-free assay could provide some information, but in vitro analysis in patient-derived cells is preferred. Furthermore, the results we obtained strongly suggest that some small mutations may unpredictably change the exon skipping propensity, implying that the exon skipping approach for in frame exons with small mutations deserves careful investigation. Furthermore, antisense targeting of small mutations might unravel splicing

regulatory motifs, because flanking introns and exons are not involved in any rearrangement such as deletions/duplications and every cis-regulatory motif like intronic splicing enhancers (ISEs) or silencers (ISSs) can play a role in the exon defining process. **Paper 1** represents the first attempt to modulate small dystrophin mutations by using both wild-type and "mutation-specific" AONs, and highlight both the peculiar splicing characteristics of mutated exons and the complexity of designing optimal AONs for exon skipping therapy toward personalized therapy.

Restoration of the reading frame in vivo

The first attempt in vivo was performed by Mann and colleagues (Mann 2001). Mdx mouse muscles (quadriceps) were injected with 1 μg of the 2'-OMe AON together with 2 μg of Lipofectin, once per week, either for 2 or 4 weeks. One week after the last injection, dystrophin was detected. Obtained dystrophin levels were rather low, therefore, to improve dystrophin levels, higher concentrations of AONs, new chemical modifications to AONs backbone (Mann 2002; Lu 2003a and 2005; Alter 2006; Vitiello 2008; Heemskerk 2009; Yokota 2009; Wu 2010) , pepide-conjugates (Yin 2008a, 2008b, 2009, 2010; Ivanova 2008; Jearawiriyapaisarn 2008, 2010; Wu 2008), octa-guanidine AONs (Wu 2009), guanine analogs (Hu 2010), nano and co-polymers (Sirsi 2005, 2008 and 2009; Williams 2006, 2008) nanoparticles (Rimessi 2009; Ferlini 2009), miscrobubbles (Alter 2009) and viral vectors using u1or u7 recombinant proteins (Goyenvalle 2004; Denti 2006; Denti 2008; Lorain 2008) have been used.

In vivo studies

AON delivery using delivery compounds

AONs have to be present in the nucleus in order to induce antisense mediated pre-mRNA splicing. While sufficient intra-nuclear delivery can generally be achieved in vitro using cationic polymers (Errington 2003), efficient AON delivery in vivo is much more complicated (Herweijer 2003). There are several barriers AONs have to cross in order to reach the nucleus, i.e. the cell membrane, the endosomal or lysosomal membrane and the nuclear membrane. Small DNA molecules diffuse through the nuclear pores into

the nucleus once they are freely present in the cytosol (Lukacs 2000). In most cases, however, AONs remain entrapped in endosomes and lysosomes (Loke 1989). Poly-ethylenimine (PEI, reviewed in (Kichler 2004)), one of the most extensively investigated cationic polymer condenses DNA and forms DNA-PEI complexes (or AON-PEI complexes). By virtue of the positive charge of the PEI-DNA (or AON) complexes, they can efficiently bind to the anionic proteoglycans on the cell surface. This results in endocytosis of the complex, whereupon the pH of the endosome drops, which leads to the protonation of PEI and osmotic swelling of the endosome, which eventually bursts, releasing the DNA (or AON) in the cytosol. As mentioned before, small macromolecules, such as AONs can efficiently diffuse to the nucleus. There are many different PEIs, with different molecular weights, either linear or branched, which all have different characteristics (Kichler 2004). The most commonly used PEI is the linear 22 kDa form. Transfection efficiencies of up to 100% can often be achieved in vitro using this PEI (Errington 2003). Unfortunately, PEI and cationic lipids are less efficient in vivo upon systemic delivery, probably because they interact with serum proteins, such as albumin, causing the dissembling of the PEI-AON complexes, resulting in exposure of the AON to degradation and rapid clearance (Dash 1999). Furthermore, cationic lipids may induce toxic effects both after systemic delivery and local injection (Kichler 2004). Aartsma-Rus and colleagues observed significant cytotoxicity in vitro after using high doses of the 22 kDA linear PEI (Aartsma-Rus 2003) and in vivo after intramuscular injection of AON-PEI complexes in mice, influx of monocytes and muscle degeneration occurred (Bremmer-Bout 2004). In the past years cationic lipids and other delivery systems have been modified for in vivo delivery of DNA and AONs. The most promising ones for delivery into muscle will be discussed here.

The most straightforward way to deliver AONs is plainly local or systemic injection of pure AONs. Unfortunately, local injection is less attractive since 30% of the body consists of muscle. After systemic injection of PS AONs, the majority is taken up by the liver within 8 hours and is rapidly cleared from the body, both in rat and mice (Graham 1998; Zhao 1998). Nevertheless, a small part of the AONs is taken up by other organs, including muscle (~5% of the total amount) (Akhtar 1997; Fluiter 2003). To obtain sufficient intramuscular concentrations, extremely high and thus toxic doses of PS AONs have to be injected. Increased uptake of pure DNA can be achieved by high-pressure injection (Liang 2004; Liu 2001; Zhang 2001). DNA is injected into an artery,

while the draining veins are temporarily occluded (e.g. by using a tourniquet), which is hypothesized to temporarily disrupt the extra cellular matrix, allowing the AON to enter the myofibers. This appears to be efficient in mice, rats and non-human primates for large muscles, such as the diaphragm and the lower limb muscles (Budker 1998; Liu 2001; Zhang 2001). However, it is unfeasible to target every muscle in the body using this method. Especially when one takes into account that repeated delivery is required, due to AON turnover. Biodistribution studies with LNA in mice revealed that the majority was cleared via the kidneys, and lower levels were detected in the muscle when compared to PS AONs (Fluiter 2003).

A potent way to increase AONs (or DNA) delivery after intramuscular injection is electroporation, which induces temporary damage to the plasma membranes, allowing influx of macromolecules (Lu 2003b), or pretreating muscle with hyaluronidase, an enzyme that hydrolyses hyaluronic acid, which is the major constituent of the extracellular matrix surrounding the muscle fibers (Favre 2000). Wells and colleagues applied electroporation and hyaluronidase treatment to transfect AONs into mdx mouse muscle (Wells 2003). This resulted in restored dystrophin expression in 28% of treated myotubes as detected by immunohistochemical analysis. Dystrophin could be detected for at least 8 weeks. Unfortunately, electroporation is accompanied by irreversible muscle damage and whole body treatment is unfeasible (Aihara 1998; Bhatt 1990).

AONs transfection efficiencies can be further improved by the application of microbubbles, such as Sono Vue, Sonazoid and Optison (Alter 2009).

As mentioned before, cationic lipid-AON complexes, such as PEI are not preferred for systemic delivery, due to their toxicity and low efficiencies. To prevent PEI from binding to serum proteins or being taken up by the liver after systemic injection, PEI can be modified by polyethylene glycol (PEG), which shields the cationic charge (PEG-PEI copolymers)(Williams 2006). This reduces PEI toxicity and increases the circulation half time, but unfortunately also decreases PEI efficiency (Kichler 2002; Papahadjopoulos 1991). In fact the amount of dystrophin that has been recovered by Williams and colleague was not enough to use the PEG-PEI copolymers for systemic delivery (Williams 2006). Lutz and colleagues have further investigated the possible use of PEG-PEI copolymers by mixing 2 KDa PEI with either 550 Da or 5 KDa PEG bound to 2OMePS resulting in a promising local delivery system (Williams 2008).

Alternatively, macromolar delivery systems can be applied, of which the pluronic gels and the nanoparticles are the most promising compounds (Hughes 2001).

Pluronic gels consist of one or a mixture of several nonionic block copolymers. The gels are liquid at room temperature, but become solid at body temperature. Therefore, the pluronic gel acts as a depot and allows prolonged, local delivery of AONs that is over 10-fold more efficient that pure AON injection (Lemieux 2000). The pluronic copolymer F127 has successfully been applied for local delivery of AONs in mdx mice (Lu 2003a). This resulted in the restoration of the dystrophin protein in over 20% of treated muscle fibers, and which could be detected for at least three months after injection. F127 is described as an inactive excipient and is already used for the delivery of numerous other drugs (von Moltke 2001). The fact that F127 becomes a solid gel at body temperature makes it a less suitable compound for systemic delivery. Biodegradable nanoparticles are small structures that either entrap AONs or bind AONs to their cationic surface (Hughes 2001). They protect the AONs to degradation and enhance transfection (Chavany 1994). However, upon degradation of the particles the extremely toxic formaldehyde is produced (Hughes 2001). Therefore, these nanoparcticles are not suitable for in vivo delivery of AONs.

Two groups tested the feasibility of exon skipping in the mdx mouse using using new nanospheres chemistries ir order to avoid side effects: the group of Lutz (Sirsi 2009) and our group (Rimessi 2009; Ferlini 2010). Sirsi and co-workers have induced specific exon 23 skipping in the mdx mouse using biodegradable poly(lactic-co-glycolic acid) (PLGA) nanospheres which are made by biocompatible compounds FDA approved and utilized in a wide variety of drug delivery applications including the encapsulation of nucleic acids. Dystrophin was rescued and the number of dystrophin positive fibers was 3.4 higher compared to naked AON injection, however these results have been achieved after intramuscular injestion (Sirsi 2009).

In **paper 2** we describe the first application of cationic core–shell nanoparticles made up of a core of polymethylmethacrylate (PMMA), surrounded by a shell bearing cationic groups. PMMA T1 nanoparticles have cationic groups, ideal for AONs' binding, covalently bound to the particles thus avoiding desorption and instability problems.

PMMA-based nanoparticles were already described as *in vivo* drug-delivery systems for the delivery of both DNA oligonucleotides and peptides. We demonstrated that the T1 nanoparticles can efficiently bind 2′OMePS AONs and that T1 nanoparticles are widely distributed in various tissues/organs including heart and skeletal muscle. We also showed that IP administration of the M23D AON adsorbed onto T1 nanoparticles induced the restoration of dystrophin protein expression in skeletal muscles and, although at lower levels, in the heart of *mdx* mice. The dystrophin rescue is associated with increased dystrophin transcript and expression of high molecular weight dystrophin protein, as shown by western blot analysis. The novel dystrophin correctly localized at the sarcolemma, as detected by immunohistochemical analysis. These results demonstrate the effectiveness of this approach both in terms of body-wide distribution and protein synthesis restoration. Moreover, dystrophin rescue was obtained using a very low dose of AON, corresponding to 1/50th to 1/80th of the routine dosage described in the literature for systemic treatments of *mdx* mice. Dystrophin synthesis is clearly induced by the T1/M23D complexes because the injection of the same dose of naked M23D did not produce any effect. The combination of slow release and depot effects, together with the protection from degradation/sequestration, afforded by this delivery system could be responsible of the very low amount of AON required for producing a functional effect. However a possible disadvantage in using PMMA nanoparticles is related to their slow biodegradability, possibly causing adverse effects due to accumulation in chronic treatments.

AON delivery without delivery compounds

Several groups have reported attempts to induce speficic exon without using any transfection reagent in mdx mice (Hu 2010; Wu 2010; Wu 2009; Heemskerk 2009; Yin 2009; Hu 2010; Ivanova 2008; Vitiello 2008; Fletcher 2006 and 2007), in the mdx/utrn$^{-/-}$ mice (Goyenvalle 2010) and in the GRMD dog (Yokota 2009).

Repeated injections of 2OMePS, PMO and PNA AONs have resulted in body wide dystrophin restoration, significantly improved serum creatine kinase values (a measure for muscle integrity) and improved muscle function in the *mdx* mouse model (Alter 2006; Fletcher 2007; Lu 2005; Yin 2010), even using repeated injections at very low doses (Malerba 2009). PMO treatment has also been tested in a dog model, where antisense-

induced exon 6 and 8 skipping resulted in dystrophin restoration and improved muscle function (Yokota 2009). Unfortunately, for each chemistry exon skipping and dystrophin levels were (much) lower or non existent in heart. A breakthrough has been achieved with peptide conjugated PMOs (pPMOs). The addition of these arginine-rich cell penetrating peptides improved exon skipping levels in all muscles, and resulted in significant exon skipping and dystrophin restoration in heart (Jearawiriyapaisarn 2008; Yin 2008). A recent report showed that repeated pPMO treatment in 4 month old mice could ameliorate the severity of the cardiomyopathy 7 months later (Jearawiriyapaisarn 2010). The pPMOs were also able to improve the phenotype and lifespan of the very severely affected double knockout mdx/utrn$^{-/-}$ mouse model (Goyenvalle 2010). However, this could only be achieved when treatment started very early (2 week old pups) and using relatively high doses of pPMO. Alternatively, non peptide dendrimeric octaguanidine moiety tags have been used to improve delivery of PMOs to skeletal muscle and heart (Vivo-Morpholino)(Wu 2009). Further improvement was achieved in *mdx* mice using a PMO containing both an arginine-rich peptide and a muscle-targeting peptide, which resulted in significant dystrophin levels in muscle and heart at very low doses (Yin 2009). Many different cell penetrating peptides are now under investigation (Ivanova 2008), and generally dystrophin restoration can be achieved with much lower doses than for unconjugated AONs (Yin 2009). However, these peptides have not yet been tested in humans. Thus, the translation into clinical trials will take more time than for 2OMePS and PMOs, which have already been tested in human subjects extensively. A disadvantage of the AON approach is that due to AON and muscle turnover, repeated injections are required. This could be circumvented by building the antisense sequence into a gene, which would allow continuous expression of the antisense sequence. The small nuclear ribonucleoproteins (snRNPs) provide very suitable tools for this, as they already consist of a RNA antisense sequence within a protein complex and are located primarily in the nucleus. Modification of U1 and U7 snRNPs that were delivered to cultured cells or to *mdx* mice indeed resulted in long term exon skipping and dystrophin restoration (over 1 year) (Denti 2006 and 2008; Goyenvalle 2004). This system also allows for the generation of bifunctional snRNPs that contain an antisense sequence and a binding motive for the hnRNP A protein, that can suppress exon inclusion (Goyenvalle 2009). The adenoassociated viral vector (AAV) system appears the method of choice to deliver these antisense genes to muscle, as it allows efficient, long term

transduction of muscle (Denti 2008; Goyenvalle 2004). However, some major hurdles need to be overcome before this approach can be tested in the clinic, e.g. the upscaling of medical grade AAV production, the immunogenicity problems (which precludes repeated injections) and the identification of ways to achieve body wide (or whole limb) delivery (van Ommen 2008).

Clinical Trials

Since exon skipping that restored the open reading frame in patients, disrupts the open reading frame in unaffected individuals, the exon skipping approach cannot be tested in healthy volunteers. Thus initial clinical trials were performed in patients using either a very low dose or local injections. The first trial was performed in Japan and used 4 weekly intravenous injections of 0.5 mg/kg PS AONs (without a 2'-O-methyl modification) targeting exon 19 in a single patient with a deletion of exon 20 (Takeshima 2006). Exon 19 skipping could be detected in the patient's lymphocytes and at very low levels in patient's muscle. A small number of dystrophin positive muscle fibers could be detected in a muscle biopsy. Since exon 51 skipping applies to the largest group of patients, AONs targeting this exon are developed for clinical application by two companies. Prosensa Therapeutics (the Netherlands) and AVI Biopharma (USA).

The hDMD mouse model has been useful for pre-clinical in vivo optimization of human AONs targeting exon 51 and 53 (Arechavala-Gomeza 2007; Popplewell 2010). Due to sequence differences between the murine and human *DMD* genes, human specific AONs generally will not result in exon skipping in mice (Bremme-Bout 2004; Heemskerk 2009). This mouse model has been also used to confirm the efficacy of AAV delivered U7 snRNPs targeting human exon 51 (Goyenvalle 2010).

Prosensa Therapeutics uses PRO051, a 2OMePS AON. Intramuscular injection of 0.8 mg PRO051 in the tibialis anterior muscle was tested in 4 Dutch DMD patients in 2006 (van Deutekom 2007). This was well tolerated and resulted in exon 51 skipping and dystrophin restoration in all patients. Similar results were obtained in 5 patients in the UK in a trial sponsored by AVI Biopharma (USA). These patients received an injection of 0.9 mg AVI-4658, a PMO, in their extensor digitorum brevis muscle, which also resulted in exon 51 skipping and dystrophin restoration in the absence of side effects (Kinali 2009). A lower dose (0.09 mg) of this AON was ineffective. Remarkably, the

number of dystrophin positive muscle fibers 64-97% vs 44-79%) and protein levels (17-35% vs 22-32% for PRO051 and AVI-4658, respectively) were very similar for both trials, although differences between the two trials and methods of analysis preclude direct comparison (Aartsma-Rus 2009b).

After this initial proof-of-concept in humans, current trials test systemic delivery of AONs. A phase I/II clinical trial sponsored by Prosensa involving 12 patients from Belgium and Sweden who received 5 weekly doses of 0.5 – 6 mg/kg PRO051 (3 patients per dose) has been completed. The treatment was well tolerated for all doses. Preliminary results are very encouraging. Dystrophin restoration was observed for each patient in a dose dependent manner (http://prosensa.eu/news/Press%20release%20WMS.pdf). An extension study were all patients are treated for 6 months with the highest dose is currently ongoing in preparation for a phase III pivotal trial where patients will be treated for a longer time to allow assessment of a functional effect.

An AVI-Biopharma-sponsored trial testing 12 weekly intravenous treatments of 0.5 – 20 mg/kg AVI-4658 is currently ongoing in the UK. Thus far treatment is also well tolerated, and while no dystrophin was observed in patients treated with the lowest doses, exon 51 skipping was observed in patients treated with 2 and 4 mg/kg and for one of the patients treated with 2 mg/kg dystrophin was detected as well (http://www.avibio.com/news_detail.php?newsId=0068). Results for the highest doses (10 and 20 mg/kg) are pending.

RNA quantification studies

The most commonly used parameter when comparing AONs is the exon skipping percentage. This is defined as the percentage of transcripts in which the targeted exon is skipped relative to the total number of dystrophin transcripts (skipped and non skipped). As anticipated, there appears to be a correlation between exon skipping percentages and dystrophin restoration in that AONs that induce high levels of exon skipping also lead to higher dystrophin levels than AONs that are less efficient (Heemskerk 2009). Thus, when optimizing AONs, assessment of exon skipping percentages is a relatively straightforward way to compare the efficiencies of different AONs. However, different ways to quantify exon skipping levels are used by different

groups, making it difficult to compare results between labs (Heemskerk 2009; Arechavala-Gomeza 2007; Yin 2008; Ivanova 2008; Lu 2005; Doran 2009; Denti 2008). Since dystrophin expression is rather low, cDNA synthesis followed by two rounds of PCR amplification (primary and nested PCRs) is the method used most, though again protocols greatly differ and the total number of amplification cycles can vary from ~50-55 (Heemskerk 2009; Yin 2008; Ivanova 2008; Lu 2005) to 65-70 (Doran 2009; Denti 2008). Only one group has recently used a single round amplification (Wu 2009 and Hu 2010 – Qi Long Lu group) to assess exon 23 skipping in the mdx mouse. In **paper 3** we reviewed the most used methods and compared them using AON treated mouse muscle derived RNA as input material in order to assess the optimal method to determine exon skipping percentages. We introduced as new methods to measure exon 23 skipping in the *mdx* mouse model a melting curve analysis (MCA) and a digital array by Fluidigm[TM]. MCA was discarded because of low sensitivity. Digital array allowed us to quantify the absolute exon skipping percentages and was used to compare the other five methods.

Using the digital array we determined that ~1100 dystrophin transcripts are present in 1 ng of mRNA and that 665 transcripts are sufficient to correctly determine the exon skipping percentage using only 30 cycles of amplification. These data match with the data published by Mortazavi and colleagues (Mortazavi 2008), in which an RNA-Seq experiment was performed in (amongst others) skeletal muscle tissue of a wild type mouse. When we compared different molecular approaches of several reports that assessed exon skipping in *mdx* mice we observed a huge difference in the number of dystrophin RNA molecules used as template ranging from 1100 to 17700 transcripts. We also show that all groups make use of too many amplification cycles and that the excessive number can lead to non reliable measurements and overestimation of skipping levels. We believe standardization is needed in order to better undestrand the outcome of different treatments inducing exon skipping.

It has been suggested that exon skipping levels assessed by RT-PCR are overestimated, since the smaller skipped products are amplified more efficiently than the bigger nonskipped fragments. However, we show in **paper 3** that for 30 amplification cycles using a single round amplification, this is not the case. When comparing skipping values obtained with absolute and relative quantification via densitometry and bioanalyzer analysis for a single round PCR, we found that skipping

values were very similar. Thus, apparently stochastic effects do not interfere with the measurements. Most of the values that fall in a 25% error margin relative to the absolute quantification results, were obtained using either bioanalyzer or densitometer. Non-overestimation is also underlined by the finding that a one round amplification (20/25/30 cycles) of isolated skipped and unskipped molecules in known skipping percentages led to a slight underestimation of real skipping percentages rather than an overestimation. However, when two rounds of amplification were used, skipping levels were generally overestimated. Nevertheless, since dystrophin is a lowly expressed gene, one round of amplification might not be sufficient when analyzing exon skipping in cultured cells or in muscles from severely affected patients. This will require further investigation. We concluded that exon skipping can be quantified using different techniques. However, quantification using a bioanalyzer or a densitometer after primary amplification seems the preferred method for *mdx* muscle tissue derived samples, since the values obtained are close to the real values for both low and high exon skipping levels. These methods are more straightforward and significantly less expensive than digital array analysis, which serves as a golden standard. It is however possible that the optimal method(s) will differ for other exons and therefore methods to quantify the skipping of other (human) exons should assessed individually, although as a more general rule exon skipping levels should not be quantified using nested PCR.

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Exon Skipping-Mediated Dystrophin Reading Frame Restoration for Small Mutations

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ABSTRACT: Exon skipping using antisense oligonucleotides (AONs) has successfully been used to reframe the mRNA in various Duchenne muscular dystrophy patients carrying deletions in the *DMD* gene. In this study we tested the feasibility of the exon skipping approach for patients with small mutations in in-frame exons. We first identified 54 disease-causing point mutations. We selected five patients with nonsense or frameshifting mutations in exons 10, 16, 26, 33, and 34. Wild-type and mutation specific 2′OMePS AONs were tested in cell-free splicing assays and in cultured cells derived from the selected patients. The obtained results confirm cell-free splicing assay as an alternative system to test exon skipping propensity when patients' cells are unavailable. In myogenic cells, similar levels of exon skipping were observed for wild-type and mutation specific AONs for exons 16, 26, and 33, whereas for exon 10 and exon 34 the efficacy of the AONs was significantly different. Interestingly, in some cases skipping efficiencies for mutated exons were quite dissimilar when compared with previous reports on the respective wild-type exons. This behavior may be related to the effect of the mutations on exon skipping propensity, and highlights the complexity of identifying optimal AONs for skipping exons with small mutations. Hum Mutat 30:1527–1534, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: exon skipping; antisense oligonucleotides; dystrophin; *DMD*

Introduction

Nonsense or frame-shifting mutations in the *DMD* gene (MIM♯ 310377) lead to Duchenne muscular dystrophy (DMD; MIM♯ 310200), a severe X-linked neuromuscular disorder due to the complete absence of functional dystrophin protein in males. By contrast, the milder allelic Becker muscular dystrophy (BMD; MIM♯ 310376) is caused by in-frame mutations that give rise to a smaller but functional protein [Hoffman et al., 1987; Monaco, 1989]. The interruption or maintenance of the dystrophin reading frame by the gene mutations explains the phenotypic differences observed in approximately 92% of the BMD/DMD cases [Aartsma-Rus et al., 2006; Koenig et al., 1989]. Although the majority of the mutations in the *DMD* gene consists of large rearrangements, an increasing number of both nonsense and small frame-shifting mutations has been widely reported, due to the improvement of the diagnostic systems [Bennett et al., 2001; Buzin et al., 2005; Flanigan et al., 2003; Hofstra et al., 2004; Roberts et al., 1993, 1994; Tuffery-Giraud et al., 2004; Whittock et al., 1997]. It is now estimated that point mutations account for nearly 30% of dystrophin mutations [Aartsma-Rus et al., 2006; Deburgrave et al., 2007].

Due to the approach of mutation-specific clinical trials, dystrophin mutation characterization meanwhile has become mandatory. For instance, only patients carrying nonsense mutations are eligible for participation in the phase I/IIa trial held by PTC Therapeutics, based on a stop codon read-through strategy [Welch et al., 2007]. Another mutation-specific approach is antisense-induced exon skipping aimed at the reframing of dystrophin transcripts. Among the various antisense types or modifications, the 2′OMePS antisense oligonucleotides (AONs) targeting exon 51 have been recently used in a pilot study in four DMD patients with very encouraging results [van Deutekom et al., 2007].

Because the majority of DMD-causing mutations are out-of-frame deletions clustered within the two major hotspot regions, the exon skipping approach has been focused on this type of rearrangements. However, it has recently been estimated that, in theory, single and double exon skipping would be applicable to 79% of deletions, 91% of small mutations, and 73% of duplications, amounting to 83% of all *DMD* mutations [Aartsma-Rus et al., 2009]. Indeed, only two nonsense mutations were approached in vitro by 2′OMePS AONs designed on the wild-type exon sequence [Aartsma-Rus et al., 2003, 2004].

Although single exon skipping for small mutations can be applied for mutations lying within in-frame exons only, many patients are still eligible for such a treatment (11% of all DMD patients; Aartsma-Rus et al., 2009). Therefore, the general aim of this study was to confirm the applicability of single exon skipping to small mutations. We also intended to evaluate whether "universal" or "private," mutation-specific AONs were more effective in inducing the exon skipping of mutated dystrophin exons, effectively impacting the design of future therapies for these patients. It is, in fact, known that small mutations can disrupt exon splicing enhancers (ESEs) and silencers (ESSs), splicing regulatory sequences devoted to the exon recognition process [Cartegni et al., 2003]. Mutations located in ESE and ESS elements may affect splicing. Consequently, a significant proportion of exonic point mutations could exert an effect completely different as would be predicted from the genetic code. Positive and negative regulatory elements play opposite and fundamental roles in a finely tuned exon recognition process [Nielsen et al., 2007], which need to be taken into account when interfering with the splicing process. Because mutations lying within ESEs may strongly affect the splicing process [Solis et al., 2008], we used this double approach to point out this relevant topic.

We have identified 54 unrelated DMD patients carrying small mutations in the *DMD* gene, of which 32 are novel. Among 24 patients carrying a mutation in an in-frame exon, we selected 5 mutations in 5 different exons to be modulated (listed in Table 1). We induced the specific exon skipping using AONs designed either on the wild-type or on the mutated sequence both in cell-free splicing assays (four exons) and myogenic cell cultures (all five exons). The predictive value of a cell-free splicing assay on exon skipping propensity was assessed to have an alternative assay in case cells from the patients were not available. The AONs design was performed on the basis of well established in silico parameters (sites for SC35 and SF2/ASF binding sites) [Aarstma-Rus et al., 2005, 2008]. This approach follows the concept of targeting exon sequences containing ESEs. It has, in fact, already been established that AONs-mediated exon skipping occurs by blocking the access of the splicing machinery to ESE sites in the exon [Aartsma-Rus et al., 2006]. Exon-internal AONs have proven to be more effective than AONs lying on the canonical splice sites [Aartsma-Rus et al., 2008]. They also reduced the possible risk of off-targeting of nonspecific sequences [Aartsma-Rus et al., 2004].

We showed that skipping efficiencies for certain mutated exons were significantly different from what has been previously reported for the respective wild-type exons. This is the first report describing AONs modulation of dystrophin exons containing point mutations through efficacy comparison of the wild-type versus the mutation-specific "private" AONs.

Material and Methods

Mutation Analysis

DNA from 54 unrelated DMD patients was extracted by QIAGEN BioRobot (Universal System 8000) from whole blood, after informed consent and for diagnostic purposes. All these patients were negative for *DMD* deletions/duplications as assessed by MLPA.

We designed a set of 79 primer pairs for single-exon amplification. Oligonucleotide sequences and amplification conditions are available upon request. The amplicons were sequenced using a 3130 automated sequencer (Applied Biosystem, Bedford, MA)

Table 1. Patients with Dystrophin Small Mutations Analyzed and Characteristics of the AONs Used in the Present Study

Patient	Mutation	n. exon	AON type	AONs sequence	AON length	% GC	Cell-free assays skipping %	SF2/ASF	SC35	SRp40	SRp55	Distance from 3'ss	Distance from 5'ss	5' Intron length	3' Intron length	Cloned intron boundaries
3	c.1132_1135dupCAGT	10	WT	guccucagcagaagaagagcc	20	55.00	14.83	4.62 (2)	3.20 (2)	3.88	2.82	100 nt	73 nt	52,717 bp	650 bp	nt 961–196; nt 1,149+98
		10	MS	guaugaacugacuggucuu	20	40.00	25.63	2.94	3.28 (3)	/	2.86	166 nt	7 nt	52,717 bp	650 bp	nt 961–196; nt 1,149+98
7	c.1912delC	16	WT	ucuuuucuagauccgcuuuuaa	22	31.82	5.10	2.24	2.46	3.87	3.07	3 nt	154 nt	7,648 bp	20,367 bp	nt 1,813–293; nt 1,992+97
		16	MS	uucugguccacugacuuauucuucag	25	36.00	5.10	3.82 (3)	3.23 (3)	3.67 (2)	3.39	78 nt	76 nt	7,648 bp	20,367 bp	nt 1,813–293; nt 1,992+97
18	c.3447_3448GG>TT	26	WT	ucuguaauucaucuggaguu	20	35.00	−8.2	3.82	3.08	3.63	3.74	131 nt	20 nt	8,606 bp	6,023 bp	nt 3,433–211; nt 3,603+206
		26	MS	cucccucaaggccuaauuc	21	47.62	−13.5	3.36	/	/	/	10 nt	140 nt	8,606 bp	6,023 bp	nt 3,433–211; nt 3,603+206
21	c.4565delT	33	WT	ccgucugcuuuuucuguaca	20	45.00	50.72	5.62	/	/	/	67 nt	68 nt	3,035 bp	5,629 bp	nt 4,519–227; nt 4,674+120
		33	MS	cuuaucccauuuccaccucag	22	40.91	19.93	2.34 (2)	2.44	4.21 (2)	3.74	31 nt	102 nt	3,035 bp	5,629 bp	nt 4,519–227; nt 4,674+120
23	c.4780delTins37	34	WT	uccauucuguagcugccag	20	50.00	9.19	/	3.31 (3)	3.27	3.74 (2)	84 nt	67 nt	5,629 bp	15,310 bp	nt 4,675–231; nt 4,845+298
		34	MS	gaucauaugacagauaugau	20	30.00	85.55	/	/	/	/	108 nt	29 nt	5,629 bp	15,310 bp	nt 4,675–231; nt 4,845+298

List of DMD patients carrying small mutations within dystrophin gene, who were selected for AONs targeting on MyoD transfected fibroblasts. Mutations were annotated on GenBank reference sequence NM_004006.1. Characteristics of the AONs used in the present study are shown. For each AON the highest value is given for each of the SR proteins; the number of nucleotides between the AONs and the 5' and 3' splice sites (SS) is also shown. The distance to the 3' and 5' splice sites is determined from the first (3' splice site) or last (5' splice site) nucleotide in the target sequence. Last column shows the nucleotide composition of the cloned exons and adjacent intron boundaries used for the cell-free in vitro splicing assay.
aMutation in patient 23 has been previously described [Tuffery-Giraud et al., 2004]. AONs = antisense oligonucleotides.

following the manufacturer's recommendations. Supp. Table S1 lists the mutations identified. Mutation numbering system is based on cDNA sequence (GenBank reference sequence NM_004006.1) in which nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Constructs

The five selected mutated exons and relative intron boundaries (shown in Table 1) were amplified from the patients' genomic DNAs using in-house protocols (available upon request) and cloned in the previously described pBG vector [Gualandi et al., 2003]. Forward primers for each amplicon were designed upstream of the branch site consensus, whereas reverse primers were located at least 100 nucleotides downstream of the donor splice site. The correct nucleotide composition of all constructs was confirmed by sequencing.

AONs Design and Synthesis

Wild-type AON design was based on the highest ESE-Finder value [Cartegni et al., 2003] (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process = home) and on the scores for SF2/ASF and SC35, paying attention to the absolute distance to the 3′ splice site as previously reported [Aartsma-Rus et al., 2005]. AONs sequences, characteristics, and chosen parameters are shown in Table 1.

The AONs were synthesized with a full-length phosphorothioate backbone and 2′-O-methyl modified ribose molecules. Oligonucleotide synthesis was carried out on an ÄKTA™ oligopilot plus 10 DNA/RNA synthesizer (GE Healthcare, Piscataway, NJ) using its trityl-on mode. The sequence was synthesized on a 2-μmol scale using Primer Support 200 loaded at 80 μmol/g (Amersham Biosciences, Piscataway, NJ) as previously described [Rimessi et al., 2009]. The final oligonucleotide was dissolved in water and filtered through a short column of Dowex 50WX8 (Na+ form, 100–200 mesh) to afford after lyophilization 0.8 μmol (40%) of target compound. The purity of the full-length desired product was confirmed by MALDI-TOF MS, ^{31}P-NMR and RP-HPLC analyses.

In Vitro Cell-Free Splicing Assay

Five in-frame mutated exons cloned into the pBG splicing vector, were transcribed in vitro using T7 RNA polymerase and α^{32}-rGTP, and purified from 5% acrylamide gel to perform in vitro cell-free splicing assays in HeLa nuclear extracts, as previously described [Gualandi et al., 2003]. All the splicing products were reverse transcribed using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were amplified using the Ex2/Ex3 oligos designed on the rabbit β-globin sequences [Gualandi et al., 2003], cloned in the pCRII vector (TA Cloning Invitrogen, Carlsbad, CA), and sequenced on ABI Prism 3130 (Applied Biosystems) to confirm the accuracy of the exon splicing process (amplification conditions and primer sequences are available upon request).

To induce exon skipping in the cell-free in vitro system, 2′OMePS AONs were used in concentrations ranging from 100 to 500 nM. The incubation lasted 3 hours. To semiquantify the amount of the skipped transcript we performed quantification on images from scanned autoradiographs. The fragments were quantified by Quantity One software (BioRad, Hercules, CA). We compared the amount of spliced products with the total amount product in the presence as well as in the absence of AON. We assumed that the total of the two transcripts (unspliced/spliced) accounts for 100%.

Myogenic Cell Cultures and AONs Transfection

Primary human fibroblasts from five patients (3, 7, 18, 21, and 23 in Supp. Table S1 and listed also in Table 1) were isolated from skin biopsies (obtained after informed consent for research purposes, Ethical Approval N. 9/2005). Cells were cultivated in high-glucose DMEM (GIBCO, Gaithersburg, MD), supplemented with 20% fetal bovine serum (FBS; GIBCO) and antibiotic/antimicotic solution (Sigma, St. Louis, MO). Myogenesis was induced by infection with an Ad5-derived, EA1-deleted adenoviral vector carrying the MyoD gene as previously described [Aartsma-Rus et al., 2003; Havenga et al., 2002; Roest et al., 1996]. All experiments were carried out using cells with a maximum of four passages. Myotubes obtained after 10–14 days of culture in differentiation medium (2% FBS) were transfected with AONs (100 nM) in the presence of polyethylenimine (ExGen500, MBI Fermentas) (2 μl per μg of AON) as transfection reagent, as previously described [Van Deutekom et al., 2007].

Myogenic Cultures RNA Studies

Forty-eight hours posttransfection, total RNA was isolated from myotube cultures (RNeasy Kit, Qiagen, Chatsworth, CA) and reverse transcribed into cDNA using random primers and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was performed on β-actin to verify cDNA synthesis and on dystrophin (primers sequence available upon request) using primers situated on several flanking exons to exclude spontaneous multi-exon skipping. For exons 26 and 34 a nested PCR was necessary to detect specific exon skipping.

PCRs were performed by five cycles of 94°C (30 sec), 63°C (30 sec), and 72°C (30 sec) plus 30 cycles of 94°C (30 sec), 62°C (30 sec), and 72°C (30 sec). PCR products were analyzed on 1.5% agarose gels. All amplified fragments were sequenced on ABI Prism 3130 (Applied Biosystems). We did not perform semi-quantitative densitometric analysis on RT-PCR products, because we adopted the Real-time quantitative assay.

Exon-Specific Real-Time Assay (ESRA)

To precisely quantify the percentage of exon skipping we set up eight novel ESRAs detecting human dystrophin exons 6, 10, 16, 26, 33, 34, 40, and 70. These exons were chosen because they are not involved in spontaneous alternative splicing events in humans. We performed the ESRAs on exons 6, 10, 16, 26, 40, and 70, considered as references, to quantify the amount of dystrophin transcript both in patients' and in control myotubes. In addition, the same ESRAs were used to measure the level of physiological exon skipping and the percentage of induced exon skipping in treated cells compared to untreated cells (internal reference). These data were confirmed using an external reference gene (β-actin gene) on cDNA samples from each myotube culture (treated and control cells). All ESRAs are based on TaqMan MGB technology, and have been designed by PrimerExpress Applied Biosystems software (primer and probe sequences are available upon request). The amount of the target sequences in respect to internal references (represented by a nonmutated dystrophin

exon) and to an appropriate endogenous control (β-actin gene) was evaluated by the comparative CT method in respect to the untreated control (ΔΔCt Method) (Applied Biosystems User Bullettin #2).

Immunofluorescence Analysis

Treated myotube cultures were grown onto coverslips and fixed in −20°C methanol at 2–6 days posttransfection, depending on the survival rate of the myotubes. Samples were incubated for 30 min in phosphate-buffered saline (PBS) additioned with 4% bovine serum albumin. All samples were labeled with a polyclonal antidystrophin antibody (H300 Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:40, washed with PBS, and revealed with a TRITC-conjugated antirabbit secondary antibody; all samples were double labeled with mouse monoclonal antibodies against desmin or developmental myosin heavy chain (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), diluted 1:10 and 1:60, respectively. After several washings with PBS, all samples were incubated with FITC-conjugated antimouse secondary antibodies (Dako, Carpenteria, CA). The slides were mounted with antifade mounting medium (Molecular Probes, Eugene, OR) and analyzed using Nikon Eclipse 80i fluorescence microscope.

Results

Small Mutations Identification

A series of 54 patients with no deletions or duplications were screened for small mutations. Identified mutations are reported in Supp. Table S1. Among the 54 mutations, 32 are novel. The mutation in patient 23 was previously described [Tuffery-Giraud et al., 2004] and has been included in Table 1 (and Supp. Table S1), as the fibroblasts of this patient were used for AON modulation.

Pathogenic mutations were identified for each patient. Mutations include splicing, frame shifting, and nonsense mutations, and 24 DMD patients show small mutations in 16 different in-frame exons. To confirm the applicability of exon skipping for small mutations occurring within in-frame exons we selected five patients to be investigated (number 3 [NM_004006.1:c.1132_1135dup in exon 10], 7 [NM_004006.1:c.1912_1912delC in exon 16], 18 [NM_004006.1:c.3447_3448delGGinsTT in exon 26], 21 [NM_004006.1:c.4565delT in exon 33], and 23 [NM_004006.1:c.4780delTins37 in exon 34]).

AONs Design

For each exon we designed AONs specific for the mutation (mutation specific, msAONs) and AONs not covering the mutations (wild-type, wtAONs) (see Table 1). AONs targeting exons 16 and 26 have been previously designed targeting the acceptor splice sites [Wilton et al., 2007]. We decided to design and synthesize novel exon 16 and 26 AONs targeting exon-internal sequences. The wtAON against exon 33 is 5 nucleotides shorter than the one reported previously [Wilton et al., 2007]. AON on exon 34 was already designed and tested, but because it did not produce any skipping [Wilton et al., 2007], we designed a novel AON closer to the 3′ splice site.

ESE-finder software showed that the mutations in exon 10 and 34 did not result in differences in predicted SR protein binding sites. In contrast, the mutated exon 16 shows two additional SR proteins binding sites caused by the mutation (among these, the highest score was for SC35 protein). In exon 33, the mutation

causes the formation of a binding site for the SC35 protein. The exon 26 mutation is responsible for the loss of two SR protein binding sites.

Taking these data into account and considering that our scope was to also include mutation-specific AONs, we were able to design msAONs for exons 10, 16, 26, and 33 according to the rules described above (see Materials and Methods section). The mutation in exon 34 consists of the insertion of a novel stretch of 37 nucleotides, and we decided to design the msAON to target the inserted sequence. This stretch does not result in significant changes in SR protein binding sites as predicted by ESE-finder analysis.

In Vitro Cell-Free Splicing Assays

The five mutated exons (10, 16, 26, 33, and 34 from patients 3, 7, 18, 21, and 23 in Table 1) were cloned into the pBG splicing vector [Gualandi et al., 2003] and analyzed using in vitro splicing assays. Splice variants were characterized and all the exon–exon junctions were sequenced (data not shown). In all cases the sequencing of the cloned splicing products demonstrated that the dystrophin mutated exons were correctly spliced between exons 2 and 3 of the rabbit β-globin gene (Fig. 1A). Antisense modulation using 2′OMePS msAONs and wtAONs was able to induce exon skipping in four out of five constructs in cell-free splicing assays at the scaled AONs concentration used (Fig. 1B). The semiquantitative method we adopted is based on the quantification of the amount of the spliced transcript (included exon) compared to the total amount of product (spliced and unspliced) in the presence of AON relative to the same ratio in the absence of AON. In a major part of the in vitro assays the exon-skipping percentage did not depend on the AON concentration (Fig. 1C). We therefore calculated the average value of the skipping percentages (Fig. 1D), which could be more easily compared to the AONs efficacy in patient-derived cells. We observed that msAON was more efficient for skipping exon 34 (85.55% msAON vs. 9.19% wtAON) and exon 10 (25.63% msAON vs. 14.83% wtAON), whereas wtAON was more efficacious for exon 33 skipping (50.72% wtAON vs. 19.93% msAON). For exon 16 we did not observe any differences in skipping efficacy between wt and msAON (about 5%), whereas we were not able to skip exon 26 in vitro (Fig. 1C and D).

Myogenic Cell AONs Transfection and Transcription Analysis

The same wt- and ms-AONs tested in the in vitro splicing system were used in cell cultures. Using both AON types we were able to detect two products representing the unskipped and skipped transcripts by both primary (exons 16 and 33) and nested RT-PCR (exon 26 and 34) in patient-derived cell cultures (Fig. 2A). In contrast, no exon 10 skipping was observed in either primary nor nested PCR (Fig. 2A), in accordance with previously published results obtained with the universal AON [Wilton et al., 2007]. These experiments were highly reproducible.

RT-PCR of both untreated and treated cells did not show any unspecific splicing product, excluding the cells carrying the exon 10 mutation, which always showed a low level of physiological exon 9 skipping. This event has also been observed in untreated control muscle cells [Aartsma-Rus et al., 2005; Reiss and Rininsland, 1994].

To precisely quantify the specific level of exon skipping as well as to detect very low skipping amounts, we used an ESRA approach for all patient-derived treated and untreated cells. The
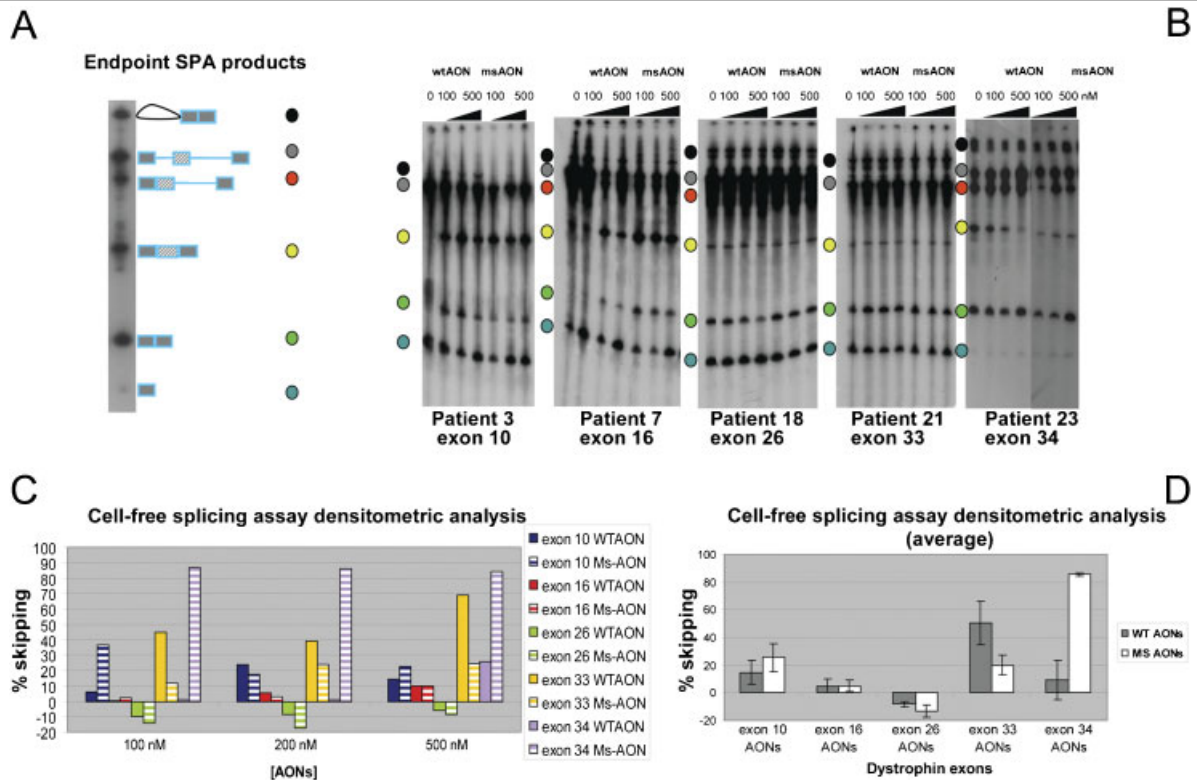
Figure 1. **A:** In vitro cell-free splicing assay endpoint (incubation time of 3 hours), with a scheme of all possible products. Colored dots represent the different products: black: lariat molecule; gray: unspliced template; red: splicing intermediate; yellow: exogenous exon spliced between β-globin exons 2 and 3; green: exogenous exon skipped transcript (β-globin exons 2 and 3); blue: β-globin exon 2. **B:** Specific exon skipping using 2′OMePS AONs in the in vitro cell-free splicing assays. wtAONs were designed on a wild-type sequence, msAONs were designed on mutated sequences. The five mutated exons (patients 3, 7, 18, 21, and 23) were in vitro spliced in the presence of scaled AONs concentrations (100, 200, and 500 nM). **C:** Graphic representation of the densitometric analysis of the exon skipping percentages obtained using the 100, 200, and 500 nM concentrations of all the different AONs. The colors and bars differentiate the skipping efficacy of each mutated exon with wt or msAONs. **D:** Graphic representation of the skipping average value considering the results obtained using all the scaled AONs concentration.

quantification was made using both adjacent dystrophin exons and β-actin as reference transcripts and in comparison to nontreated cells. The skipping percentages are shown in Fig. 2B. Comparing the Ct values obtained with proximal and distal dystrophin exons (β-actin as reference), a 5′-3′ transcriptional unbalance emerged, with 5′ transcripts more abundant in respect to the 3′ transcripts (data not shown). We therefore quantified the skipping percentage of the target exons using adjacent exons as references.

In patient 3 (c.1132_1135dup in exon 10) ESRA assay using exons 6 and 16 as references showed exon 10 skipping levels of 10.9% with msAONs, but only 2.4% of skipping with the wtAON. In contrast, patient 23 (c.4780delTins37 in exon 34) revealed skipping of 6.5% with wtAON and only 1.6% with msAON, using exons 26 and 40 as references. For patients 7 (c.1912delC in exon 16), 18 (c.3447_3448delinsTT in exon 26) and 21 (c.4565delT in exon 33) exon skipping levels were comparable for wt and ms AONs. Exon 33 skipping levels were very high: 58.8% (wtAON) and 58.3% (msAON) using exons 10 and 26 as references, exon 16 skipping levels were lower: 13.2% (wtAON) and 15.8% (msAON), using exons 10 and 20 as a reference, and skipping levels for exon 26 were 1.6% (wtAON) and 2.3% (msAON), with exon 10 and 16 as reference exons. ESRA analysis, assessing the skipping level through comparison with many references, provided robustness to the data, allowed us to detect the exon 10 skipping not visible by

RT-PCR, and identified a low level (less than 0.5%) of spontaneous skipping of the mutated exons in all untreated cells.

In conclusion, the difference in skipping efficiency is significant for exons 10 ($p = 0.0002$) and for exon 34 ($p = 0.0017$), because msAON induced higher levels of exon 10 skipping, and wtAON was more efficient for skipping exon 34 (Fig. 2B). In contrast, the skipping efficiencies for exons 16 and 26 did not vary significantly.

Immunofluorescence Analysis

Immunofluorescence analysis of desmin (data not shown) and developmental myosin heavy chain (Fig. 3A) was performed to define the differentiation stage of the Myo-D transformed fibroblasts. Double-labeling with antidystrophin antibodies revealed the rescue of the protein in all patient-derived cells treated with wtAONs or msAONs. Blinded observation as to the treatment state was performed to quantify the cytoplasmic fluorescence counting fluorescent cells/number of nuclei. The number of dystrophin positive myotubes varied significantly from 5 to 15%. Lowest levels (5%) were observed for cells with the mutation in exon 10 and treated with the wtAON, for exon 26 treated with both wt and msAONs, and for exon 34 treated with msAON. This corresponds to the lower levels of exon skipping observed for these AONs (Fig. 2B). Dystrophin localized at the sarcolemma in all transfected myotubes (Fig. 3A and B).
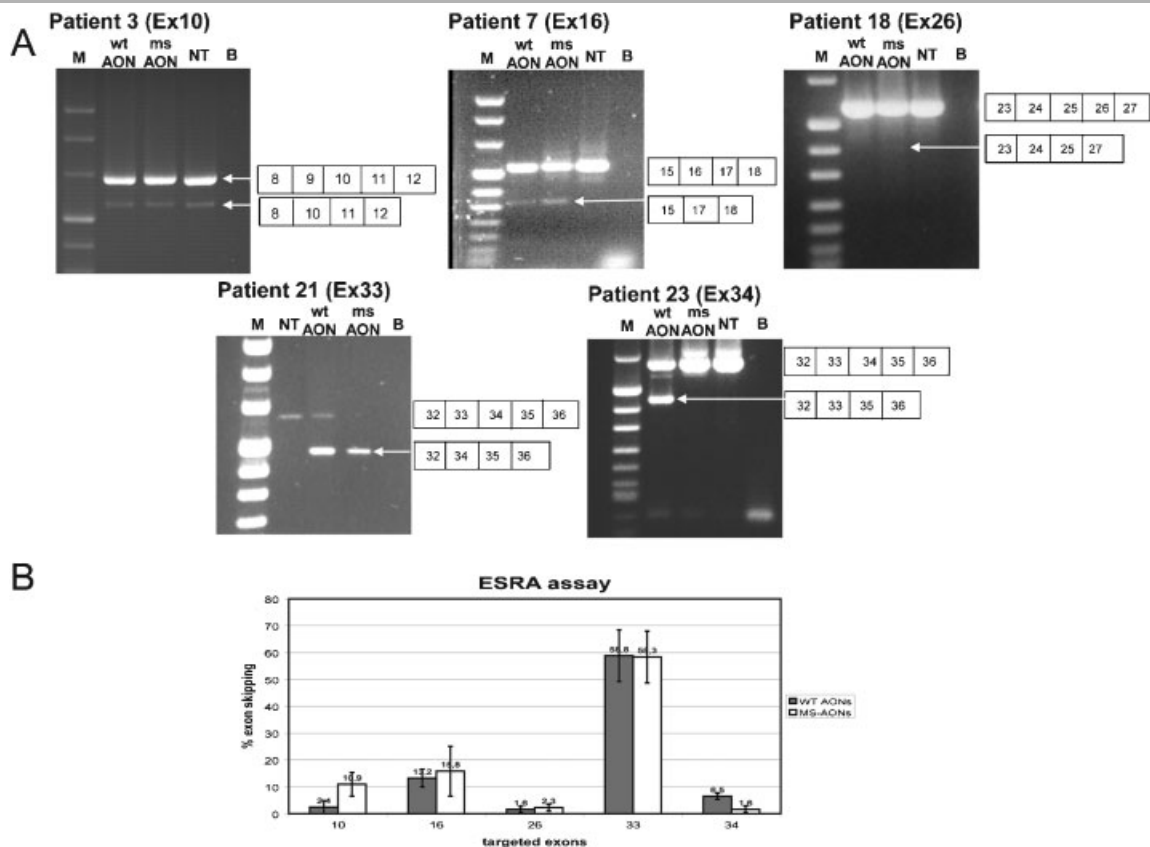
Figure 2. **A:** RT-PCRs of AONs-induced exon skipping in myogenic cells. Exons 10, 26, and 34 (patients 3, 18, and 23, respectively in Table 1) skipping was detected by nested RT-PCR, for the other two exons (30 and 33), primary RT-PCR only was performed. M is molecular weight marker VIII, B: blank sample. **B:** Graphic representation of dystrophin exons 10, 16, 26, 33, and 34 skipping quantitative differences in patient-derived AONs-treated Myo-D transformed fibroblasts in comparison with untreated cells calculated by Real-time assay (ESRA). Histograms represent the percentage of specific exon skipping using either wtAONs (gray bars) or msAONs (white bars) of treated cells with respect to untreated cells.

Discussion

The aim of our study was to induce AON-mediated exon skipping of in-frame exons containing small mutations. We tested 2′OMePS AONs targeting both wild-type and mutated sequences to evaluate the skipping performance and to compare the skipping efficiency between ms and wtAONs. We took advantage from a large cohort of patients we had identified to carry small mutations. We modulated both in cell-free and in patient-derived cells 5 dystrophin "in frame" exons (10, 16, 26, 33, and 34) containing five different small *DMD* mutations.

For the five selected dystrophin exons, the splicing was effectively reproduced in cell-free assays; however, AONs modulation was successful only for exons 10, 16, 33, and 34, whereas exon 26 could not be skipped. For exons 10 and 34 msAONs worked better, whereas for exons 16 and 33 the wtAONs produced higher skipping levels. These data suggest that cell free splicing assays might be used to test the exon skipping propensity. However, because the exon composition/length as well as the flanking intronic regions are crucial for the in vitro splicing [Eperon et al., 1988], peculiar mutations like the long stretch included in the mutated exon 34 could hamper the optimal AONs identification in the in vitro assay. Therefore, it cannot be considered a general tool for optimizing the design of the AONs.

The fact that our test produced quite different results compared to patient-derived myogenic cells underlines this. For the mutated exons 16 and 33, we obtained similar levels of exon skipping with both wt and msAONs, comparable to what was previously described for wt exons [Wilton et al., 2007].

The mutation involving exon 10, not altering the splicing enhancer quality/distribution and therefore expected to be silent in term of skipping propensity, makes the mutated exon vulnerable to skipping with a 10.9% efficacy with msAON; slightly higher than described for the wt exon in myogenic cells testing [Wilton et al., 2007]. A possible explanation of this partially positive finding is that the mutation changes the exon 10 conformation. Although encouraging, the ESRA on exon 10 does not allow us to determine whether a contextual exon 9 skipping occurs [Wilton et al., 2007]. However, we underline that exon 9 skipping is a physiological, very common alternative splicing event, and it might therefore be very difficult to sort out its exclusion from the transcript. In addition, as exon 9 is in-frame, this exon skipping is unlikely to be detrimental.

Exon 26 was considered an exon difficult to skip (ratio <10%) [Wilton et al., 2007]. In our hands, both wtAON and msAON induced indeed a similar, very poor skipping level (1.6 and 2.3%, respectively). This finding does not seem to reflect the expected increase of exon 26 skipping propensity linked to the loss of the two SR protein binding sites due to the mutation, as predicted by the ESE finder program.

The insertion in exon 34 is difficult to analyze. Although it appears not to have any modifying influence on splicing factors
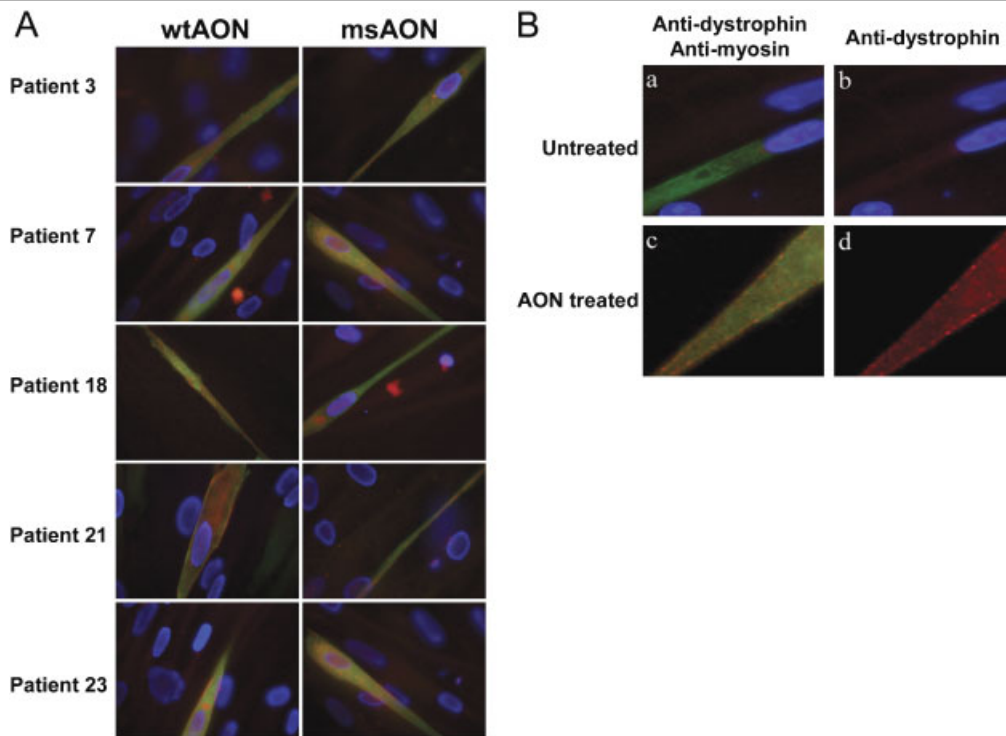
Figure 3. Immunofluorescence analysis of dystrophin in patients' cells. **A:** Double labeling with antimyosin heavy-chain (green) and dystrophin (red) antibodies clearly demonstrated the rescue of dystrophin expression in Myo-D tranformed fibroblasts of all treated patient-derived cells. More than 50% of the AON-treated cells in all examined fields showed cytoplasmic green fluorescence, which was quantified as fluorescent cells/number of nuclei. Among these, 5–15% myotubes were dystrophin positive. High magnification (B, d) demonstrates the correct protein localization at the sarcolemma. wtAON indicates antisense oligonucleotide designed on the wild-type exon, msAON indicates antisense oligonucleotide designed on the mutation. **B:** Untreated and AON treated patient's cells stained with antimyosin heavy-chain and antidystrophin antibodies. No dystrophin signal is detected in untreated cells (a, b), whereas dystrophin membrane signal is observed upon the induced exon skipping in treated cells (c, d).

binding sites, obviously this long stretch may alter the exon skipping propensity in other ways. In fact, this mutation made the exon skipping propensity in myogenic cells increase, which is in contrast with previously published data [Wilton et al., 2007]. Indeed, we induced the skipping of the mutated exon 34 in 6.5% of the dystrophin transcript using the wtAON in patient-derived cells. A possible explanation is that the insertion/deletion mutation (insertion of 37 nucleotides and deletion of 1 nucleotide) enhances the exon 34 skipping propensity by enlarging the distance between motifs recognized by the SR proteins and other splicing proteins. Alternatively, the RNA secondary structure change could affect the recognition of the mutated exon, even though the accessibility of the splice sites is the same for wild-type and mutated exon 34 (data not shown).

In conclusion, we induced the specific exon skipping in four cell-free assays and in all five myogenic cells out of five selected mutated exons. When comparing the two different assays it was demonstrated that, in the absence of patient-derived cells availability, the cell-free assay could provide some information, but in vitro analysis in patient-derived cells is preferred. Furthermore, the results we obtained strongly suggest that some small mutations may unpredictably change the exon skipping propensity, implying that the exon skipping approach for in frame exons with small mutations deserves careful investigation. Furthermore, antisense targeting of small mutations might unravel splicing regulatory motifs, because flanking introns and exons are not involved in any rearrangement such as deletions/duplications and every

cis-regulatory motif like intronic splicing enhancers (ISEs) or silencers (ISSs) can play a role in the exon defining process.

Notably, AON-mediated skipping of these five exons was never accompanied by other unspecific splicing products, such as the induction of cryptic splicing. The only alternatively spliced product was exon 9 skipping that was also observed in control myotubes. It is plausible (and very interesting) that fusion introns, resulting from deletion or duplication junctions, may influence adjacent exon recognition and therefore splicing accuracy.

Despite the promising results obtained using the exon skipping approach, another crucial point should be considered: the possible effect of the lack of skipped exons on the dystrophin protein. Isolated deletions of exons 10 and 34 are described causing a BMD or only high CK phenotype [Fokkema et al., 2005]. An isolated deletion of exon 16 causes high CK only [Schwartz et al., 2007; and personal observation]. For exons 26 and 33, however, no isolated deletions are reported in the Leiden Open Variation Database LOVD database [Fokkema et al., 2005; White and den Dunnen, 2006]. Therefore, the skipping of these exons may be of low predictive value on the resulting phenotype(s). It might also be possible that such deletions have not been identified yet due to the fact that these deletions do not result in any dystrophic phenotype. However, they all belong to the spectrin-like region (exon 10 to the repeat 1, exon 16 to the repeat 3, exon 26 to the repeats 7–8, exon 33 and 34 to the repeats 11 and 12), and, according to the literature data, their skipping is expected to have mild/BMD-like consequences on the protein function.

The data presented here report the first attempt to modulate small dystrophin mutations by using both wild-type and "mutation-specific" AONs, and highlight both the peculiar splicing characteristics of mutated exons and the complexity of designing optimal AONs for exon skipping therapy toward personalized therapy.

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Cationic PMMA Nanoparticles Bind and Deliver Antisense Oligoribonucleotides Allowing Restoration of Dystrophin Expression in the *mdx* Mouse

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For subsets of Duchenne muscular dystrophy (DMD) mutations, antisense oligoribonucleotide (AON)-mediated exon skipping has proven to be efficacious in restoring the expression of dystrophin protein. In the *mdx* murine model systemic delivery of AON, recognizing the splice donor of dystrophin exon 23, has shown proof of concept. Here, we show that using cationic polymethylmethacrylate (PMMA) (marked as T1) nanoparticles loaded with a low dose of 2′-*O*-methyl-phosphorothioate (2′OMePS) AON delivered by weekly intraperitoneal (IP) injection (0.9 mg/kg/week), could restore dystrophin expression in body-wide striated muscles. Delivery of an identical dose of naked AON did not result in detectable dystrophin expression. Transcription, western, and immunohistochemical analysis showed increased levels of dystrophin transcript and protein, and correct localization at the sarcolemma. This study shows that T1 nanoparticles have the capacity to bind and convoy AONs in body-wide muscle tissues and to reduce the dose required for dystrophin rescue. By immunofluorescence and electron microscopy studies, we highlighted the diffusion pathways of this compound. This nonviral approach may valuably improve the therapeutic usage of AONs in DMD as well as the delivery of RNA molecules with many implications in both basic research and medicine.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked inherited muscle degenerative disorder mainly caused by frame-disrupting mutations due to large rearrangements in the dystrophin gene.[1] DMD boys are affected by both severe skeletal muscle wasting and dilated cardiomyopathy. The milder allelic form of the disease, Becker muscular dystrophy, is due to in frame mutations, which preserve a shortened but functional protein. This observation has suggested that converting a DMD phenotype to a less severe condition may represent a possible therapeutic approach for DMD. In this context, restoration of dystrophin synthesis by exon skipping during pre-mRNA splicing by means of antisense oligoribonucleotide (AON) could potentially be applicable for up to 90% of DMD patients.[2] Indeed, this approach has been successfully applied to the *mdx* mouse model, both *in vitro* and *in vivo*, to normal and DMD patients' myogenic cells, to the transgenic humanized DMD mouse, and to the canine model of DMD. These results have therefore generated optimism about the feasibility of developing effective therapy approaches for DMD.[3–5] Indeed, it was recently shown that intramuscular injection of 2′-*O*-methyl-phosphorothioate (2′OMePS) AONs in four DMD patients was associated with relevant local rescue of dystrophin synthesis.[6] Notwithstanding these encouraging results, the systemic administration of AONs is limited by their cellular uptake and nontoxic effective doses. The main challenge in this field is therefore to improve the delivery of AONs to all tissues affected by the disease, including skeletal and cardiac muscles, and to reduce the effective therapeutic dose in view of life-long treatment.[6–8] In particular, to obtain exon skipping in the heart is of importance, because cardiomyopathy is currently the leading

cause of death in DMD patients. Very recently two independent studies described a high yield of dystrophin protein in the heart by systemic delivery of morpholino oligomers conjugated with cell-penetrating peptide containing arginine.[9,10] However, the nondegradable nature of phosphorodiamidate morpholino oligomer still raises concerns over their safety after extended systemic applications.[11] Experimental evidence in the *mdx* model indicates that engineered AONs can efficiently reach the cardiac muscle also when cloned into recombinant adeno-associated virus vectors.[12] However, the intrinsic immunogenicity of virus-associated proteins may impair the efficacy of viral-mediated gene inoculations preventing prolonged transgene expression. Indeed, whereas mice may be more tolerant to initial exposure to AAV, recent data demonstrated immune-mediated loss of recombinant adeno-associated virus persistence in the DMD dog model and in patients participating to a phase 1/2 clinical trial of β-hemophilia.[13,14]

The use of synthetic nonviral vectors based on polymeric systems is a versatile and safe approach for the *in vivo* delivery of bioactive molecules, including plasmid DNA, oligonucleotides, and peptides. They increase their stability and shelf life in biological fluids, improving their efficacy. Over the past decade, several polymeric delivery systems, such as liposomes, copolymers, nano-, and micro-spheres, have been developed.[15] The compounds are encapsulated inside the polymeric matrix and released *in vivo* by a combination of diffusion and polymer degradation. However, following encapsulation and release, labile drugs, such as DNA and proteins, may undergo significant degradation accompanied by a reduction in drug activity.[16] Moreover intracellular drug release from the polymeric matrix may be too slow to be effective. In fact, particles could be removed from the intracellular environment before much of the payload has been released.[17]

To achieve an effective binding, cationic micro- and nanospheres consisting of biodegradable polymers (poly(lactic-co-glycolic acid)) were therefore obtained in which cationic surfactants are able to adsorb drug onto particles' surface (*i.e.*, cetyltrimethylammonium bromide) or cationic polymers (*i.e.*, polyethyleneimine).[18,19] However, this approach suffers from an inherent surface instability due to the occurrence of cationic surfactant desorption, thus ultimately leading to reproducibility problems and even toxic side effects. To overcome these problems, we designed and prepared a novel type of cationic core–shell nanospheres (T1), made up of a core of polymethylmethacrylate (PMMA), surrounded by a shell bearing cationic groups. PMMA T1 nanoparticles have cationic groups, ideal for AONs' binding, covalently bound to the particles thus avoiding desorption and instability problems. PMMA-based nanoparticles were already described as *in vivo* drug-delivery systems for the delivery of both DNA oligonucleotides and peptides.[20,21]

Anionic and cationic PMMA–based nanoparticles similar to the T1 sample used in this study, were already shown to be very promising delivery systems for protein and DNA vaccines or for modified peptide nucleic acids as the particle/bioactive molecules are readily taken up by the cells where they efficiently release the delivered drug, are safe in mice and nonhuman primates, even after multiple administration of high doses, and slowly biodegradable.[22–24] This knowledge prompted us to evaluate

T1 nanoparticles as alternative vehicles to deliver charged RNA-like AONs and to induce dystrophin rescue with improved efficiency and/or with more durable effect in *mdx* mice. We indeed demonstrate that T1 nanoparticles bind 2′OMePS oligoribonucleotides and have a body-wide distribution following IP administration. This was accompanied with dystrophin restoration both in skeletal muscles and in the heart. This rescue persisted up to 6 weeks after the last injection. Using T1 nanoparticles, the effective dose of AON was highly reduced (2.7 mg/kg) when compared to those used in previous studies on naked AONs delivery (120–240 mg/kg).[25,26] Our results encourage further studies on T1 or other novel nanoparticles to evaluate applicable therapeutic employment for AON delivery in DMD.

RESULTS

T1 nanoparticles and AON loading experiments

T1 nanoparticles (diameter measured by scanning electron microscope 417 nm, Z-potential +46.5 mV, surface charge density 155 μmol/g) were obtained by emulsion polymerization (**Figure 1a**). They were made of a core of PMMA, surrounded by a shell bearing cationic groups. Loading experiments to test loading capacity, depending on the physico-chemical properties of the T1 nanoparticles, such as surface charge density (155 μmol/g) and surface area, showed that T1 nanoparticles (1 mg/ml) adsorbed onto their surface 2′OMePS M23D oligoribonucleotide in the concentration range of 10–100 μg/ml. The hydrophobic nature of the 2′OMePS oligoribonucleotide is responsible for its strong
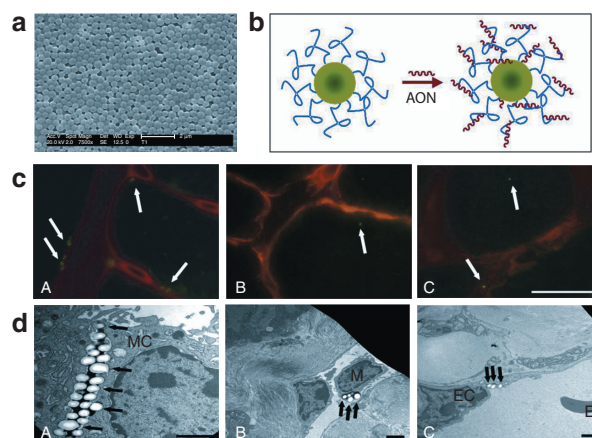

Figure 1 T1 nanoparticles characteristics and biodistribution. (a) T1 nanoparticle scanning electron microscope image showing the nanoparticles size (diameter 417 nm). (**b**) Schematic representation of the lipophilic interaction between antisense oligoribonucleotide (AON) molecules and the surface quaternary ammonium groups onto nanoparticles. (**A**) between two mesothelial cells of the peritoneal side, and inside the cytoplasm of both (**B**) macrophages in lymphatic vessels and (**C**) endothelial cells in blood vessels (arrows). (**c**) Biodistribution of T1-Fluo nanoparticles by fluorescence microscope analysis in group 3 *mdx* mice. A, diaphragm; B, skeletal muscle (gastrocnemius); C, heart. Tissue sections were labeled with antinidogen antibody (red staining). Isolated nanoparticles are detected inside the cytoplasm or close to the basement membrane of the muscle fibers (arrows). Bar = 20 μm. (**d**) Biodistribution of T1 nanoparticles by electron microscope analysis. The diaphragm shows the presence of several nanoparticles (See **Supplementary Materials and Methods**) EC, endothelial cell; M, macrophage; MC, mesothelial cell.

Table 1 *Mdx* experiments schedule

Group (no. of animals)	Formulations dose/injections	1st Injection (day)	2nd Injection (day)	3rd Injection (day)	1st Killing (day)	2nd Killing (day)
1 ($n = 6$)	T1/M23D 2.5 mg/45 µg	0	7	14	21 ($n = 4$)	60 ($n = 2$)
2 ($n = 2$)	M23D 45 µg	0	7	14	21 ($n = 2$)	
3 ($n = 3$)	T1 2.5 mg	0	7	14	21 ($n = 1$)	60 ($n = 2$)
4 ($n = 6$)	NT	NT	NT	NT	21 ($n = 3$)	60 ($n = 3$)

NT, not treated.
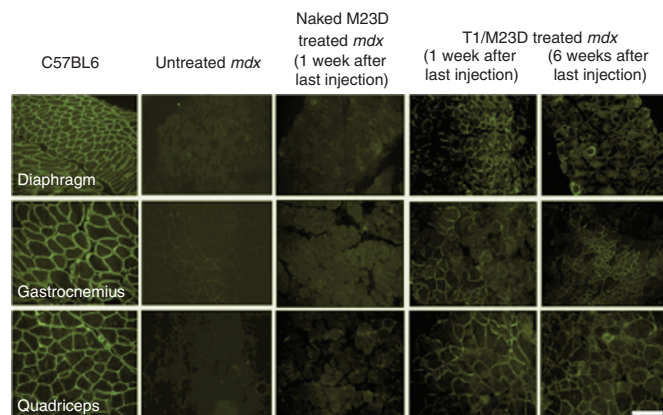
Figure 2 Immunohistochemical findings in *mdx* skeletal muscles. Dystrophin immunolabeling in muscle fibers. Representative fields of cross sections from C57BL6 wild type, untreated *mdx*, naked M23D-treated *mdx*, and T1/M23D complexes treated *mdx* mice, diaphragm, gastrocnemius, and quadriceps skeletal muscle labeled with a polyclonal antidystrophin antibody, demonstrating absence of dystrophin in *mdx* mice untreated and treated with M23D naked AON and restoration of immunolabeling in several groups of muscle fibers after treatment with T1/M23D complexes analyzed 1 and 6 weeks after the last injection. The total injected amount of AON per animal, both naked or nanoparticles-combined, was 135 µg. Bar = 40 µm.

lipophilic interaction with the surface quaternary ammonium groups and with the matrix of the nanoparticles (**Figure 1b**). M23D adsorption on T1 nanospheres was a highly reproducible process with a loading efficiency, representing the percentage of bound versus unbound oligonucleotides, ranging from 20 to 100%. In particular, the adsorption efficiency was high at low AON concentration and it slightly decreased at high AON concentration, leading to the assembling of AON/nanoparticles complexes with 18 µg/mg of loading value (data not shown).

Nanoparticles body-distribution

In order to monitor body distribution, three *mdx* mice (group 3 in **Table 1**) were treated via IP injections with fluorescent AON-free T1 nanoparticles and analyzed 1 and 6 weeks after last injection, obtaining similar results. Fluorescence analysis was performed on spleen, liver, heart, gastrocnemius, diaphragm, and quadriceps. In diaphragm, nanoparticles were detected close to the mesothelium (**Figure 1c**, A). Single particles were found intracellular in several myofibers of gastrocnemius and in the heart (**Figure 1c**, B and C). The number of particles/mm^2 was higher in diaphragm when compared to gastrocnemius and quadriceps (about 10 and 2 particles/mm^2, respectively). Transmission electron microscope examination confirmed the presence of nanoparticles in all tissues
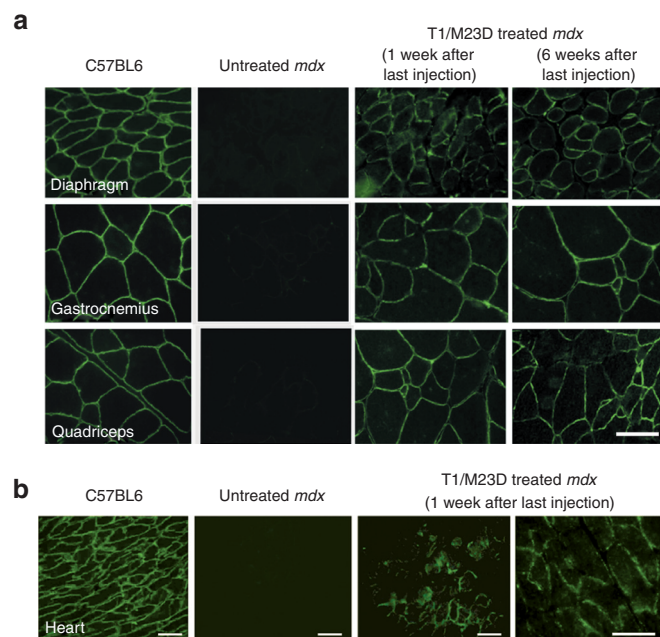

Figure 3 Immunohistochemical findings in *mdx* skeletal muscles and heart. (a) Dystrophin appears correctly expressed at the membrane of muscle fibers of T1/M23D-treated *mdx* mice both 1 and 6 weeks after last treatment. The intensity of labeling in rescued dystrophin-positive muscle fibers is reminiscent of normal muscle. No dystrophin labeling is detected at the sarcolemma of untreated *mdx* myofibers. Bar = 20 µm. **(b)** Immunohistochemical analysis of dystrophin in heart from C57BL6, untreated *mdx*, and T1/M23D-treated *mdx* mice. Restoration of dystrophin immunolabeling is detected in focal areas of cardiac muscle of mice treated with T1/M23D complexes, 1 week after last injection. When compared with normal cardiac muscle, the dystrophin immunolabeling appears discontinuous at the membrane of treated cardiomyocytes. Bar = 50 µm.

examined (**Figure 1d**). T1 nanoparticles appeared as electron-translucent round structures with an expected size of 500 nm. Nanoparticles were found both in the cytoplasm of circulating macrophages in lymphatic vessels and inside endothelial cells of blood vessels (**Figure 1d**, B and C).

Immunohistochemical analysis of dystrophin

In all skeletal muscles from mice treated with the T1/M23D complexes, dystrophin expression was restored in a significant number of fibers. The immunolabeling pattern was characterized by clusters of dystrophin-expressing fibers (**Figure 2**). Restored dystrophin localized correctly at the sarcolemma, and the intensity of labeling was comparable to the wild type (WT) muscle fibers (**Figure 3a**). However, in some groups of

fibers the labeling appeared heterogeneous. We found an average of 40, 40.27, and 45% of dystrophin-expressing fibers with a labeling covering 90–100% of the perimeter, in diaphragm, gastrocnemius, and quadriceps, respectively; the percentage of myofibers with a labeling ranging from 50 to 90%, was 44.2% in diaphragm, 55.3% in gastrocnemius, and 45.5% in quadriceps. Moreover, 10% in diaphragm, 3% in gastrocnemius, and 4% in quadriceps of myofibers showed a discontinuous pattern or a labeling that covered <50% of the perimeter. Immunohistochemical analysis of dystrophin in cardiac muscle of all T1/M23D-treated *mdx* mice examined 1 week after last injection revealed the presence of groups of dystrophin-expressing cardiomyocytes in different areas of the heart (**Figure 3b**). Dystrophin was absent in the heart of T1/M23D-treated mice killed 6 weeks after last injection (data not shown) and in control *mdx* mice (**Figure 3b**).

Quantitative analysis of dystrophin-positive fibers was performed in skeletal muscle of *mdx* mice treated with T1/M23D complexes, while cardiac muscle was not evaluated; the count of dystrophin-positive fibers in skeletal muscle excluded myofibers with a labeling that covered <50% of the perimeter or with a discontinuous pattern. The average of positive-muscle fibers varied between different skeletal muscles (**Figure 4a**). The number of dystrophin-positive

fibers found in *mdx* mice treated with T1/M23D complexes was significantly increased when compared to *mdx* mice treated with the same dose of naked M23D or to control *mdx* mice, untreated or treated with T1-Fluo AON-free nanoparticles. *mdx* mice examined 1 week after last injection showed the highest number of dystrophin-positive fibers in diaphragm and quadriceps and the lowest in gastrocnemius. In *mdx* mice examined 6 weeks after the last injection, the highest number of dystrophin-positive fibers was detected in gastrocnemius and the lowest in diaphragm and quadriceps.

Western blot

By western blot analysis the expression of dystrophin was clearly detected in gastrocnemius, quadriceps, and diaphragm from all *mdx* mice groups treated with T1/M23D complexes, examined both 1 and 6 weeks after last injection, but undetectable in the heart as well as in *mdx* mice treated with naked M23D or untreated (**Figure 4b**). Two different antibodies against dystrophin produced similar results. The size of the protein was indistinguishable from the full-length dystrophin of the normal muscle, as expected for a protein translated by a single exon 23 skipped transcript. No other lower molecular weight products were seen.
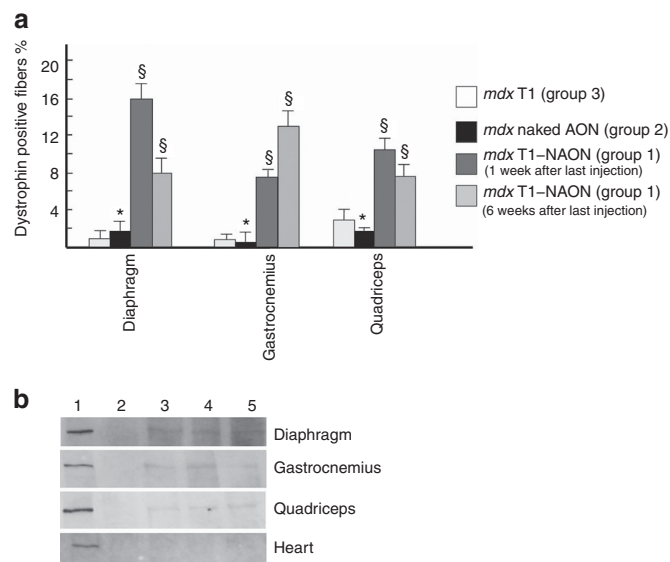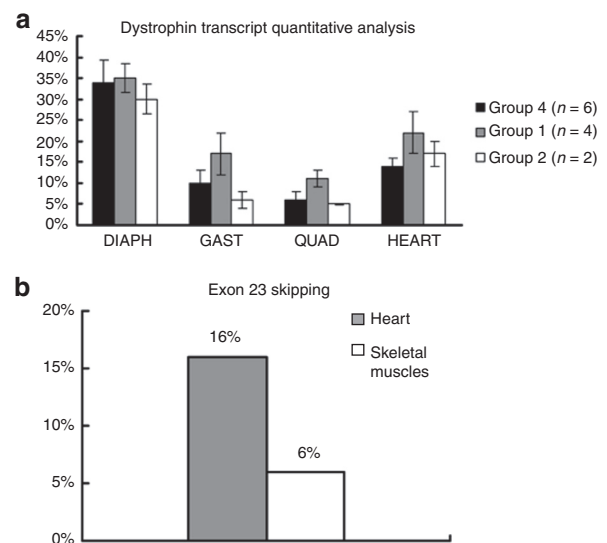

Figure 4 Positive fibers counting and western blotting. (**a**) The statistical analysis shows a significant increase of dystrophin-positive fibers in diaphragm, quadriceps and gastrocnemius of *mdx* mice treated with T1/M23D complexes (group 1) if compared to *mdx* mice treated with M23D naked AON (group 2) or with T1 nanoparticles (group 3; see **Table 1**). The average of dystrophin-positive myofibers varies among different skeletal muscles and the mice examined 1 and 6 weeks after the last injection. Data represent mean ± SD. $^{§}P < 0.05$. $^{*}P > 0.05$. (**b**) Dystrophin immunoblot using DYS2 antibody shows restored expression of the protein in diaphragm, gastrocnemius, and quadriceps muscle from T1/M23D complexes treated *mdx* (lanes 3 and 4: 1 week after last injection, lane 5: 6 weeks after last injection, group 1 in **Table 1**), while no protein is detected in *mdx* untreated control (lane 2). Dystrophin protein is undetectable in heart from both untreated and treated *mdx* mice. Diaphragm, gastrocnemius, quadriceps, and heart from normal mice are used as positive controls, 1/30th of protein loaded in the other lanes (lane 1). AON, antisense oligoribonucleotide; T1, cationic polymethylmethacrylate.

Figure 5 Transcript and exon skipping quantification. (**a**) Quantification of dystrophin transcript in skeletal and cardiac muscles from *mdx* treated and untreated animals in respect to wild type mice (considered as 100%). Dystrophin transcript expression was evaluated by relative-quantitative Real-Time PCR using β-actin as endogenous control. Histograms represent dystrophin transcript level calculated by the mean of exons 7, 22 and 25, $2^{-\Delta\Delta CT}$ values (expressed as percentage) in different skeletal muscles (diaphragm, quadriceps, and gastrocnemius) and heart. A total of 26 experiments has been performed (column represents the mean values). The analysis was performed on *mdx* mice from three different groups killed 1 week after the last injection: group 1 ($n = 4$; grey column), group 2 ($n = 2$; white column), and group 4 ($n = 6$; black column). (**b**) Percentage of exon 23 skipping determined by Real-time PCR in cardiac and skeletal muscles from *mdx* treated animals in comparison with untreated mice. The analysis was performed on group 1 mice ($n = 4$) in respect to group 4 mice ($n = 3$), all killed 1 week after the last injection. The percentage of exon 23 skipped transcript ($2^{-\Delta\Delta CT}$ values) calibrated on exon 7, was evaluated in three different experiments and appeared to be clearly assessable in the cardiac muscle (16%; grey column) as well as in the skeletal muscles, represented by gastrocnemius and quadriceps (6%; white column).

Transcription studies

Quantitative evaluation of T1/M23D treatment on general dystrophin transcriptional level. In order to quantify differences in the amount of dystrophin transcript both in skeletal and cardiac muscle from untreated, T1/M23D- and naked M23D-treated animals, we performed a quantitative analysis using exon specific real-time assays (ESRAs). This demonstrated that untreated *mdx* mice showed a generally lower transcription level in respect to the amount of the transcript (considered as 100%) detected in the WT mice, therefore used as control. In addition, the transcription level in *mdx* follows a muscle-specific pattern, being the higher level of messenger in diaphragm (34% of the WT) and heart (14%), whereas lower levels were seen in gastrocnemius (10%) and quadriceps (6%) (**Figure 5a**). The *mdx* mice group 1 (**Table 1**), treated with T1/M23D compound and killed 1 week after last injection, showed a significant increase of dystrophin transcription levels in the heart (80% more transcript), gastrocnemius, and quadriceps (70% and 50% more, respectively), although at different extent among treated mice (**Figure 5a**). No effect was observed in the diaphragm, where transcription level remained comparable in treated and untreated animals. The *mdx* mice group 2, treated with naked M23D, did not show significant difference in the dystrophin transcription level in comparison with untreated *mdx* mice (**Figure 5a**).

Exon 23 skipped transcript quantification. The percentage of exon 23 skipped transcript, calibrated to exon 7 and taking as endogenous control mouse β-actin gene, appeared to be clearly assessable in the cardiac muscle (16%) as well as in the skeletal muscles (6%) of T1/M23D-treated *mdx* mice analyzed 1 week after last injection (**Figure 5b**). In addition, the correct exon 22–exon 24 junction was assessed by sequencing the nested PCR product (See **Figure S2**). Quantitative analyses were also performed on the treated *mdx* mice killed 6 weeks after last injection; however, the transcription levels were comparable to those of untreated *mdx* mice, and the exon 23 skipped transcript was undetectable.

DISCUSSION

In this work, we describe the first application of cationic core–shell nanoparticles as effective nonviral vehicles for the delivery of charged AONs *in vivo*. We demonstrated that the T1 nanoparticles can efficiently bind 2′OMePS RNA-like oligonucleotides. We also demonstrated that T1 nanoparticles are widely distributed in various tissues/organs including heart and skeletal muscle. We also showed that IP administration of the M23D AON adsorbed onto T1 nanoparticles induced the restoration of dystrophin protein expression in skeletal muscles and, although at lower levels, in the heart of *mdx* mice. The dystrophin rescue is associated with increased dystrophin transcript and expression of high molecular weight dystrophin protein, as shown by western blot analysis. The novel dystrophin correctly localized at the sarcolemma, as detected by immunohistochemical analysis. These results demonstrate the effectiveness of this approach both in terms of body-wide distribution and protein synthesis restoration.

Moreover, dystrophin rescue was obtained using a very low dose of AON, corresponding to 1/50th to 1/80th of the routine dosage described in the literature for systemic treatments of *mdx* mice.[25,26] Dystrophin synthesis is clearly induced by the T1/M23D

complexes because the injection of the same dose of naked M23D did not produce any effect. The combination of slow release and depot effects, together with the protection from degradation/sequestration, afforded by this delivery system could be responsible of the very low amount of AON required for producing a functional effect. The above suggestion is supported by the well-known hydrophobic nature of phosphorothioates,[27] which is responsible for the strong lipophilic interactions of oligonucleotides with quaternary ammonium groups and matrix of the nanoparticles.

Because a moderate toxicity is documented for PS-AONs, even in view of chronic treatment, the possibility of obtaining sustainable effects by using a very low dose is certainly realistic.

The second interesting result is the dystrophin rescue obtained in the heart, as shown in treated *mdx* mice killed 1 week after the last injection. Although we are aware that the amount of dystrophin rescue in the heart is low, we should consider that it has been obtained by 1/80th of the dose previously administered for systemic delivery of 2′OMePS.[25] It is also known that other AON molecules as phosphorodiamidate morpholino oligomer-AONs do not reach the heart spontaneously but only when conjugated with cell-penetrating peptides.[9,10,26] Therefore, despite the low amount of rescue as well as the lack of its persistence after 6 weeks, the T1/M23D complex represents the first conjugate able to improve the 2′OMePS oligomer uptake by systemic administration.

Although delivery of 2′OMePS was demonstrated to be improved by using synthetic cationic copolymers of polyethyleneimine and poly(ethylene glycol), this delivery was performed only locally because systemic administration of these copolymers may present toxic effects.[3,28–30]

We have also set up a novel quantitative analysis (ESRA) of dystrophin transcripts that provided the identification of a muscle-specific pattern of transcription in untreated *mdx* mice in comparison with WT mice. We used both β-actin and adjacent dystrophin exons as reference transcribed regions. The ESRA analysis allowed us both to identify a wide increase of transcription levels after treatment in the different muscles, including heart, and to quantify the dystrophin exon 23 skipped transcript in the heart (16%) and in skeletal muscles (6%). The increased amount of dystrophin transcript in T1/M23D-treated *mdx* mice could be due to an enhanced stability of skipped transcript and/or to the inhibition of nonsense mediated decay. The difference between heart and skeletal muscle exon 23 skipped transcript amounts is remarkable. Furthermore, it is also evident that there is a discrepancy between transcript and protein rescue, being inversely proportional in heart versus skeletal muscle. Although explaining this peculiar but relevant finding requires further studies, there are data showing that both the transcription and translation behavior varies between heart and skeletal muscle. This may be due to *cis/trans* acting tissue specific factors able to destabilize or stabilize the transcripts or more generally to the different transcription and translation efficiency in both tissues.[31,32]

Histological analysis showed no apparent tissue damage in any of the organs examined, such as liver, kidney, and spleen (**Figure S1**).

A possible disadvantage in using PMMA nanoparticles is related to their slow biodegradability, possibly causing adverse effects due to accumulation in chronic treatments. Furthermore,

T1 nanoparticles might not be appropriate for intravenous usage, because the possibility to form small aggregates, expected by using >2.5 mg of nanoparticles.[24] For these reasons and in order to improve the potential for such a novel approach, we are currently testing different nanoparticles, varying in size, RNA binding affinity, and bioerosion propensities.

In summary, our results show that IP administration of a low dose of specific AON–nanoparticle complexes can effectively restore dystrophin synthesis in both skeletal and cardiac muscle. Nanoparticles are therefore eligible as candidate delivery systems for RNA molecules supporting further investigations in particular for DMD AON-mediated therapeutics.

MATERIALS AND METHODS

AON synthesis. M23D(+07-18) (5′-GGCCAAACCUCGGCUUACCUG AAAU-3′) AON against the boundary sequences of the exon and intron 23 of mouse dystrophin gene, contains 2′-*O*-methyl modified RNA and full-length phosphorothioate backbone.[33] Oligonucleotide synthesis was carried out on an ÄKTA oligopilot plus 10 DNA/RNA synthesizer (GE Healthcare, Milano, Italy) using its trityl-on mode. The sequence was synthesized on a 2-µmol scale using Primer Support 200 loaded at 80 µmol/g (Amersham Biosciences, Milano, Italy). Commercial 2′-*O*-methyl phosphoroamidites (Proligo, Boulder, CO) were dissolved to a nominal concentration of 50 mmol/l in anhydrous acetonitrile (CH_3CN) and activated with a 0.25 mol/l solution of 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole (Proligo) in CH_3CN. The final detritylation was achieved using a 0.1 mol/l aqueous solution of NaOAc (pH 3.0). Crude dimethyltryptamine protected and detritylated oligonucleotide were purified by an ÄKTAbasic ultra physical contact liquid chromatography system using an Amersham Biosciences Resource RPC 3 ml column eluted under a gradient of CH_3CN in 0.1 mol/l triethylammonium acetate (pH 8).

The final oligonucleotide was dissolved in water and filtered through a short column of Dowex 50WX8 (Na^+ form, 100–200 mesh) to obtain after lyophilization 0.8 µmol (40%) of target compound. The purity of the full-length desired product was evaluated by MALDI-TOF MS, ^{31}P-NMR and RP-HPLC analyses.

T1 nanoparticles loading experiments. For phosphorothioate modified RNA-like oligonucleotide (2′OMePS-AON) adsorption experiments, 1.0 mg of freeze-dried nanospheres were suspended in 1.0 ml of 20 mmol/l sodium phosphate buffer (pH 7.4) and sonicated for 15 minutes. The appropriate amount of a concentrated aqueous solution of 2′OMePS-AON was then added to reach the final concentration (10 ÷ 100 µg/ml). The experiments were run in triplicate (SD ≤ 10%). The suspensions were continuously stirred at 25 °C for 2 hours. After microfuge centrifugation at about 18,000 rpm for 15 minutes, quantitative sedimentation of the AON–nanoparticle complexes was obtained and aliquots (10 ÷ 50 µl) of the supernatant were withdrawn, filtered on a Millex GV_4 filter unit and diluted with sodium phosphate buffer. Finally, UV absorbance at $\lambda = 260$ nm was measured. Adsorption efficiency (%) is calculated as 100 × (administered AON) – (unbound AON)/(administered AON).

Animals. All experiments were performed on male *mdx* mice (C57BL/10ScSn-Dmd*mdx*/J) or age-matched WT male mice (C57BL/10SnJ). All procedures were approved by the Animal Experimentation Ethics Committee. Mice were housed in temperature-controlled rooms (22 °C) with a humidity of 50% and a 12:12 hour light–dark cycle. Mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

IP injections of T1–AON complexes, T1-Fluo-AON free, and naked AON. T1 nanoparticles toxicity tests were performed both in mice and in murine/human cells using up to 5 mg/injection (IP) in animals

and 10 mg/ml in cells. No toxicity was observed both in *in vivo* and in *in vitro* systems (A. Caputo, Department of Histology, Microbiology, and Medical Biotechnology, personal communication, September 2006).

mdx mice (8–10 weeks of age) were IP injected with 250 µl of T1/M23D complex, containing 45 µg of M23D AON and 2.5 mg of T1 nanoparticles (thought to represent a well-tolerated dose both for multiple IP and for IV administration) dissolved in sterile unpreserved saline solution (0.9% sodium chloride), or 250 µl containing 45 µg of M23D dissolved in unpreserved saline solution, and monitored according to approved NIH and University guidelines. The complex suspension (250 µl) was slowly injected through the abdominal skin into the peritoneal cavity using an insulin syringe fitted with a 28-gauge needle. One group of *mdx* mice (n = 6) (group 1) received three identical injections of T1/M23D complex at days 0, 7, and 14. One group of *mdx* mice (n = 2) (group 2) received three identical injections of M23D naked AON at the same times. Controls were age-matched *mdx* mice injected at days 0, 7, and 14 with fluorescent T1 AON-free nanoparticles (n = 3) (group 3) and untreated *mdx* mice (n = 6) (group 4) as negative controls. The total amount of the administered M23D AON was 135 µg/animal. **Table 1** summarizes treatments and killing of mice analyzed in the present work.

Harvest of tissues. At 1 week after the third injection, four *mdx* mice of group 1, two *mdx* mice of group 2, one *mdx* mouse of group 3 and three *mdx* mice of group 4, were killed and diaphragm, gastrocnemius, quadriceps, and cardiac muscles were isolated, blotted dry, trimmed of external tendon, pinned to Parafilm-covered cork, snap frozen in liquid N_2-cooled isopentane, and stored at −80 °C until further processing. Muscles from the two remaining *mdx* mice of group 1 together with the control *mdx* mice, two from group 3 and three from group 4, were harvested 6 weeks after the last injection (16–18 weeks of age). Liver, kidney, and spleen were also harvested.

Fluorescent microscope analysis of nanoparticles distribution. Seven-micrometer thick frozen sections of liver, spleen, heart, diaphragm, quadriceps, and gastrocnemius skeletal muscles from *mdx* mice of group 3 (**Table 1**) were labeled with a rabbit antinidogen antibody (Calbiochem, San Diego, CA), selected as marker of basal lamina, incubated with an antirabbit tetramethyl rhodamine iso-thiocyanate–conjugated secondary antibody (DAKO, Glostrup, Denmark), washed several times with phosphate-buffered saline and observed with a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments, Firenze, Italy). Serial sections, obtained from three different levels of each organ at 100-µm intervals were observed at 100× and images recorded with a Nikon digital camera.

Transmission electron microscope study of nanoparticles distribution. Liver, spleen, kidney, diaphragm, gastrocnemius and quadriceps skeletal muscles, and heart were fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer for 3 hours and with 1% osmium tetroxide in Veronal buffer for 2 hours, dehydrated with ethanol and embedded in Epon E812.

Ultrathin sections were stained with lead citrate and uranyl acetate and observed in a Philips EM 400 transmission electron microscope (FEI, Hillsboro, OR), operated at 100 kV.

Immunohistochemical analysis of dystrophin. Seven-micrometer thick frozen sections of heart, diaphragm, quadriceps, and gastrocnemius skeletal muscle fragments from *mdx* treated with M23D AON T1-conjugated complex (group 1), M23D naked AON (group 2), T1-Fluo AON-free (group 3), and untreated *mdx* mice (group 4), were labeled with a polyclonal antidystrophin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100, incubated with an antirabbit tetramethyl rhodamine iso-thiocyanate–conjugated secondary antibody (DAKO), washed several times and observed with a Nikon Eclipse 80i fluorescence microscope.

Evaluation and quantification of dystrophin-positive fibers. Dystrophin immunolabeled transverse sections from WT, treated, and untreated *mdx*

mice were analyzed with a Nikon Eclipse 80i fluorescence microscope at 20× and images were taken with a fixed exposure time (0.5 seconds) using an high-resolution CCD camera (Nikon). Images were analyzed by NIS-Element BR2.20 (Nikon) imaging program and the threshold intensity for each tissue was determined on sections of WT mice. Because in T1/MD23 treated *mdx* mice groups of fibers showed a heterogeneous pattern, the percentage of the perimeter truly labeled by the antibody was determined for each fiber by using the AnalySIS program (Soft Imaging System, Muenster, Germany). The count of dystrophin-positive fibers excluded myofibers with a labeling that covered <50% of the perimeter or with a discontinuous pattern. The number of dystrophin-positive fibers was evaluated on three different levels, 300 μm apart from each other, in the diaphragm, gastrocnemius, quadriceps, and on eight serial sections, at 100-μm intervals, of the heart. At least 3,000 muscle fibers from the diaphragm and gastrocnemius and 5,000 from the quadriceps muscles, respectively, obtained from three different levels of tissue blocks for each sample, were studied for statistical evaluation; data were analyzed according to Mann–Whitney test, and the criterion for statistical significance was $P < 0.05$.

The counts were performed blind to sample identity and the relative patterns were confirmed by an independent observer in order to unbias the observation.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting analysis. Twenty-micrometer thick frozen muscle sections were homogenized with a lysis buffer containing 7 mol/l urea, 2 mol/l thiourea, 1% amidosulfobetaine-14, and 0.3% dithioerythritol, and then centrifuged at 1,500*g* for 10 minutes. Protein concentration was determined in the supernatants with the Bradford method. Aliquots of proteins from normal *C57BL/10* mice (10 μg) and from muscles of treated or untreated *mdx* mice (300 μg) were loaded onto a 6% T sodium doedecyl sulphate–polyacrylamide gel and separated by electrophoresis. Samples were transferred to a nitrocellulose membrane overnight at 75 V. The membrane was blocked with nonfat dried milk for 60 minutes at room temperature and incubated overnight at 4 °C with the specific antibody DYS2 (a mouse monoclonal antibody to the carboxy terminal region of dystrophin, 1:100, NovoCastra, Newcastle, UK) or H-300 (a rabbit polyclonal antibody to the internal region of dystrophin, 1:200, Santa Cruz Biotechnology). After intervening washes, the membrane was incubated with horseradish peroxidase–conjugated goat antimouse or antirabbit IgG diluted 1:40,000 or 1:30,000, respectively. Immunocomplexes were visualized with the ECL Advance Western Blotting Detection Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RNA studies. Total RNA was purified from muscle biopsies of WT ($n = 2$), untreated ($n = 6$), and treated *mdx* mice ($n = 8$), by using RNeasy kit (Qiagen, La Jolla, CA) and reverse transcribed into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Frankfurt, Germany). Six novel ESRAs detecting *mdx* exons 7, 8, 22, 23, 25, and 56, were specifically developed for this study. These exons were chosen because they do not undergo spontaneous alternative splicing. ESRA on exons 7, 22, 23, and 25 were used to quantify the percentage of exon 23 skipping in treated mice. Dystrophin transcript quantification was performed by comparison with the β-actin gene on each isolated muscle from both treated and control *mdx* mice. All these ESRAs are based on TaqMan technology and have been designed by PrimerExpress Applied Biosystems software (Applied Biosystems). Primers and probes sequences are available upon request. The amount of target sequences in respect to appropriate endogenous control (β-actin gene) was evaluated by the comparative CT method with respect to the endogenous β-actin control (ΔΔCt Method) (Applied Biosystems User Bulletin no. 2). Nested RT-PCR was performed as described in ref. 25, skipped transcript was analyzed by sequencing (ABI PRISM 3130 Automated Sequencer; Applied Biosystems).

SUPPLEMENTARY MATERIAL
Figure S1. Morphological analysis of liver, kidney, and spleen from *mdx* mice treated with T1/M23D complex.
Figure S2. Nested RT-PCR analysis of dystrophin mRNA in muscle tissues of wild type, untreated, and T1/M23D-treated *mdx* mice.
Materials and Methods.

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