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# Genetic Therapy for Duchenne Muscular Dystrophy: Principles and Progress

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## 1. Introduction

This chapter focuses on the gene therapy advances made in relation to Duchenne muscular dystrophy and discusses principles and perspectives of strategies currently being developed. The chapter explains the genetic mutations that cause Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) and the differences between the two are discussed in relation to disease severity. The histopathological features of DMD are explained and discussed in the context of available animal models for DMD. There are various genetic therapeutic options available for the treatment of DMD, and the progress of each therapeutic approach is promising. A number of specific areas for the treatment of DMD are comprehensively presented, alongside in-depth description of the genetic biology of muscular dystrophy.

## 2. Muscular dystrophies

Muscle-related proteins build the structural network of muscle, and disruption of this link can cause muscle wasting and progression of numerous types of muscular dystrophy. Muscular dystrophies are a heterogeneous group of genetic disorders caused by different forms of mutations in various genes related to muscles.

### 2.1 Duchenne and Becker muscular dystrophy

The most common forms of muscular dystrophy are Duchenne and Becker muscular dystrophy (DMD, BMD). DMD and BMD are X-linked recessive muscle-wasting disorders affecting the skeletal musculature, resulting from mutations in the gene encoding dystrophin, which is a cytoskeletal protein in muscle fibres. Dystrophin protein interacts with the intracellular and extracellular dystrophin associated protein (DAP) complexes. Mutations in dystrophin gene can cause severe muscular wasting.

Different mutation types in exon/ intron regions of dystrophin gene or promoters causes various forms of dystrophinopathies (Giliberto et al., 2004). Dystrophin deficiency leads to disruption of the dystrophin associated protein (DAP) complexes, as do mutations in DAP genes in other forms of congenital and autosomal muscular dystrophy.

DMD affects 1:3500 newborn males worldwide, and patients exhibit severe, progressive muscle weakening. From about the age of 4 years, youngsters affected by DMD suffer bouts of recurrent damage and regeneration of skeletal muscle, leading eventually to muscle wasting and weakness, wheelchair-dependence, and life-threatening cardiac and respiratory complications. This debilitating disease is associated with loss of dystrophin expression caused generally by frameshift gene deletions and duplications, or by nonsense point mutations in the dystrophin gene.

In BMD, muscle pathology is generally milder than in DMD, and can even be virtually asymptomatic. The milder BMD phenotype is caused by the continued expression of truncated but partially functional dystrophin proteins in the affected muscles. This phenotype commonly arises due to mutational events which delete central rod domain elements, but nevertheless maintain the open reading frame upstream and downstream of the mutation boundaries. Thus different mutation types across exon/intron regions of the dystrophin gene can give rise to either DMD or BMD, depending upon the deletion boundaries.

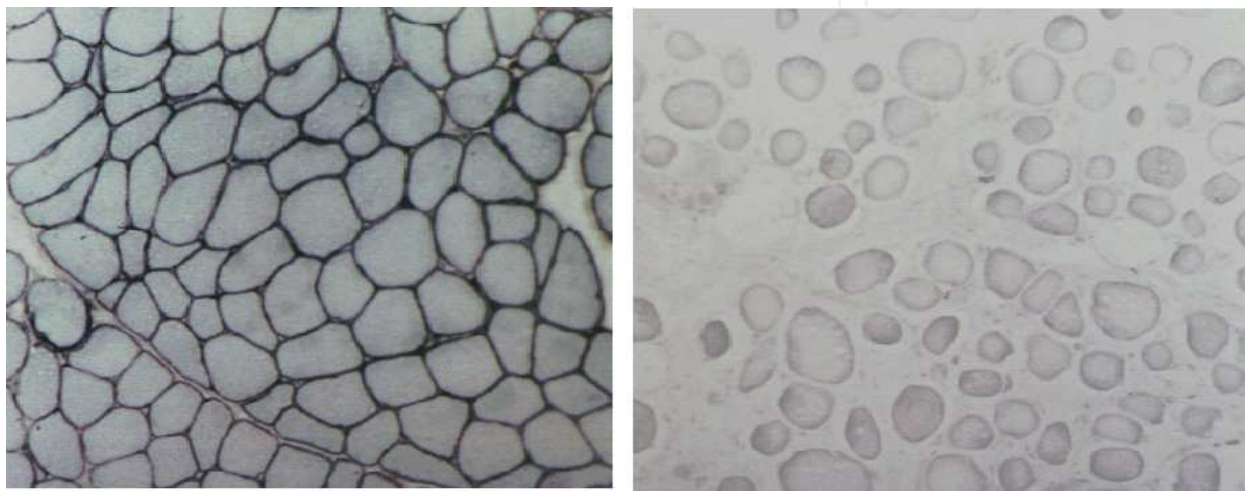
BMD can be related to large in-frame deletions, including examples where up to 46% of the dystrophin-coding sequence has been deleted (Acsadi et al., 1991). Despite the large gene deletion, BMD patients have a mild phenotype due to partial expression of an internally-deleted but highly functional dystrophin protein. Clinical phenotype of BMD pathology is discriminated by mild, intermediate and severe types resulting from different exon deletions (Chao et al., 1996). Around 20% of normal dystrophin expression is seen as the milder BMD phenotype (Beggs et al., 1991).

### 2.1.1 Histopathological features of DMD

Clinical symptoms of DMD become apparent in patients of three to five years of age, while fetal DMD muscle is histologically normal (Biggar, 2006). The lack of dystrophin causes DAP dissociation, followed by disruption of the transmembrane link between extracellular matrix and the cytoskeleton. This causes severe muscle damage along with progressive fibrosis and muscle fibre loss. Lack of dystrophin in DMD muscles is believed to compromise the integrity of the sarcolemma, leading to calcium influx and myofibre necrosis. Central nucleation and uneven size of fibres are observed in early stages of dystrophinopathies due to the muscle necrosis followed by muscle degeneration and regeneration (Bell et al., 1968, Bradley et al., 1972). In dystrophin-negative muscles, fibres are lost (necrosed) and degeneration of the fibres proceeds, and they are eventually replaced by adipose and fibrous connective tissue, followed by atrophy (**Figure 1**) (Blake et al., 2002).

In addition, an increased number of proteasomes is found in necrotic and regenerative muscles in DMD, leading to muscle fibre degradation (Kumamoto et al., 2000). Muscle membrane becomes abnormally permeable, leading to fragility and leakiness of the muscle cells. The muscle weakness results in loss of independent ambulation, usually by the age of 12 (Emery, 2001). DMD patients eventually die from intercostal muscle weakness and respiratory failure in their early twenties (Emery, 2001). DMD patients also develop cardiac dysfunction with cardiomyopathy, resulting in heart failure and progressive dilated cardiomyopathy (Emery, 2001).

There is also evidence that dystrophin deficiency is associated with aberrant signal transduction from influx of divalent cations such as calcium due to abnormal membrane permeability in DMD muscle cells (Ruegg et al., 2002). Abnormality of calcium homeostasis is found due to structural defects in the ryanodine receptor (RyR) 1, calcium release channel in the sarcoplasmic reticulum, leading to increase of intracellular  $\text{Ca}^{2+}$  in the dystrophic muscles (Bellinger et al., 2009). Increased intracellular  $\text{Ca}^{2+}$  can activate calpain, which has a role in proteolysis and increases reactive oxygen species (ROS). Calpain cleaves intracellular proteins including titin, nebulin, desmin, troponin, tropomyosin and many kinases and signalling molecules and it increases protein breakdown (Allen et al., 2005). This can cause protein and membrane damage (Whitehead et al., 2006).



Normal muscle shows even size/diameter distribution of muscle fibres with dystrophin expression localised at the sarcolemma membrane. Lack of dystrophin protein expression in DMD causes muscle damage, which is accelerated by eccentric contraction of the muscle membrane. Eventually, muscle fibres are lost (necrosed) and replaced by adipose and fibrous connective tissue.

Fig. 1. Dystrophin immunohistochemistry in normal (left panel) and DMD muscle (right panel).

Damaged muscle membranes are demonstrated by staining with extracellularly applied labelled endogenous extracellular proteins such as albumin, immunoglobulin (Ig) G and IgM (Blake et al., 2002). The already permeable membrane in DMD becomes more permeable after mechanical stress or electric stimulation. Cardiomyopathies occur as a result of cardiac muscle damage in DMD. Myocardium is replaced by fat and connective tissue in dystrophinopathies. Clinical symptoms of cardiomyopathies appear after 10 years of age in DMD, and are present in all patients over 18 years (Finsterer et al., 2003). It has been currently reported that 20% of BMD and 50% of DMD patients eventually die due to cardiac failure (Finsterer et al., 2003). In the early stages of DMD, focal myofibre necrosis initially leads to muscle stem cell activity and tissue regeneration, via activation of so-called satellite cells. As the disease progresses, however, the capacity to regenerate muscle becomes impaired owing to depletion of stem cells and fibrosis of tissues, leading finally to severe muscle wasting. This leads to inflammation of DMD muscles, as a result of inflammatory cells such as CD4 and CD8, followed by muscle necrosis (Blake et al., 2002).

Creatine kinase (CK) level is one of the indicators for DMD pathology. CK level in DMD in affected boys is elevated at birth, to 50 to 100 times the novel level, and gradually declines in the late stages of the disease (Emery, 2001).

### 2.1.2 Animal models of DMD

The development of a treatment for any disease relies on the use of appropriate animal models to test the efficacy, deliverability, dosing regimen and toxicology *in vivo* having established the therapeutic potential *in vitro*.

#### 2.1.2.1 Dystrophin deficient *mdx* model

The *mdx* mouse originates from the C57BL/10 colony and does not express the dystrophin protein due to a nonsense mutation (CAA to TAA) in exon 23 of the dystrophin gene (Sicinski et al., 1989). It shows similar pathological symptoms to human DMD patients. However the pathology of *mdx* mice is less severe compared to the human disease, because of effective regeneration of damaged muscles, which has not been observed in human DMD patients (Turk et al., 2005). In *mdx* mice, the event of degeneration/regeneration is ongoing throughout the life of the animal but peaks between the ages of 3-8 weeks (Tanabe et al., 1986). It has been demonstrated that the *mdx* mouse has a reduced life span and progressive dystrophic muscle histopathology compared to the wild-type C57BL/10 mouse (Chamberlain et al., 2007). Moreover, aged *mdx* mice are susceptible to muscle tumours, similar to human alveolar rhabdomyosarcoma (Chamberlain et al., 2007). Inflammatory cells such as CD4 and CD8 are found in dystrophin-deficient muscles followed by muscle necrosis, peaking at 4-8 weeks old of *mdx* mice (Blake et al., 2002). In dystrophic *mdx* mice, elevated CK level was also found in the serum (Bulfield et al., 1984).

#### 2.1.2.2 Canine muscular dystrophy model

The golden retriever muscular dystrophy (*GRMD*) dog was the first characterized canine model of DMD (Cooper et al., 1988, Kornegay et al., 1988, Valentine et al., 1992). It has been reported that *GRMD* dogs eventually die due to cardiomyopathy. *GRMD* has been identified as complete dystrophin deficiency with higher genotypic/phenotypic similarity to human DMD disease than that of the *mdx* mouse model. Complete loss of dystrophin is the result of a nonsense mutation in the 3' consensus splice site of intron 6, leading to skipping of exon 7 and alteration of the reading frame in exon 8, thereby inducing clinical symptoms similar to human DMD (Chamberlain et al., 2007). The Beagle-based *CXMD* canine model was also identified as a DMD model in Japan (Shimatsu et al., 2003). It has been reported that limb muscle abnormality appear after 2 months of age and the dogs eventually die mainly due to cardiomyopathy (Valentine et al., 1988). *CXMD* dog models show longer life span due to slower progression of muscle wasting compared to *GRMD* dogs (Willmann et al., 2009). Progress of cardiomyopathy in the *CXMD* dog is also milder than in *GRMD* (Yugeta et al., 2006).

#### 2.1.2.3 Hypertrophic Feline muscular dystrophy model

Hypertrophic feline muscular dystrophy (*HFMD*) is exhibited in cats (Blake et al., 2002). The creatine kinase (CK) level is increased at the age of 4-5 weeks and development of severe muscle hypertrophy is shown resulting in muscle necrosis and regeneration. However, it is not considered to be a good model for human DMD since it does not induce muscle fibrosis and muscle wasting (Gaschen et al., 1992).

## **2.2 Other muscular dystrophies**

### **2.2.1 Muscular disorders caused by mutations in membrane proteins**

Mutations in integrin have been shown to cause congenital muscular dystrophies. Integrin, membrane protein, binds to laminin. Caveloin-3, which is localised to muscle cell membrane interacts with  $\beta$ -dystroglycan and mutations in Caveloin-3 cause the autosomal dominant form of Limb Girdle muscular dystrophy 1C (LGMD1C), rippling muscle disease and hyperCKemia (Betz et al., 2001, Carbone et al., 2000).

### **2.2.2 Muscular disorders caused by mutations in extracellular matrix proteins**

Mutations in laminin  $\alpha$ -2, one of the extracellular matrix proteins, can give rise to congenital muscular dystrophy 1A (MDC1A) (Helbling-Leclerc et al., 1995).  $\alpha$  and  $\beta$  form of dystroglycan can interact with laminin and dystrophin, respectively. Mutations in  $\alpha$ -dystroglycan have been reported in the muscle-eye-brain disease, Walker-Warburg syndrome (WWS) and a type of congenital MD (Longman et al., 2003). In mice, mutations in the dystroglycan gene can be embryonically lethal (Williamson et al., 1997). Disruption to the transmembrane related sarcoglycan-sarcospan complex leads to various types of LGMD. Sarcoglycan interacts with biglycan, which also binds to  $\alpha$ -dystroglycan and as well as to collagen VI. Mutation in collagen VI gives rise to Ullrich syndrome and Bethlem myopathy (Camacho Vanegas et al., 2001).

### **2.2.3 Muscular disorders caused by mutations in intracellular proteins**

Mutation in calpain 3, which is a calcium-dependent protease, gives rise to LGMD 2A. Mutations in several sarcomeric proteins lead to LGMD 2. Heterogeneous chromosomal mutations result in LGMD. There are two types of LGMD called type 1 and type 2, which have autosomal dominant or autosomal recessive mutations, respectively. The symptoms are not as severe as DMD or BMD, but weakness of proximal limb and trunk muscles is exhibited (Lovering et al., 2005). Mutations in syntrophin and dystrobrevin display mild forms of skeletal and cardiac muscle disease.

### **2.2.4 Muscular disorders caused by mutations in nuclear proteins**

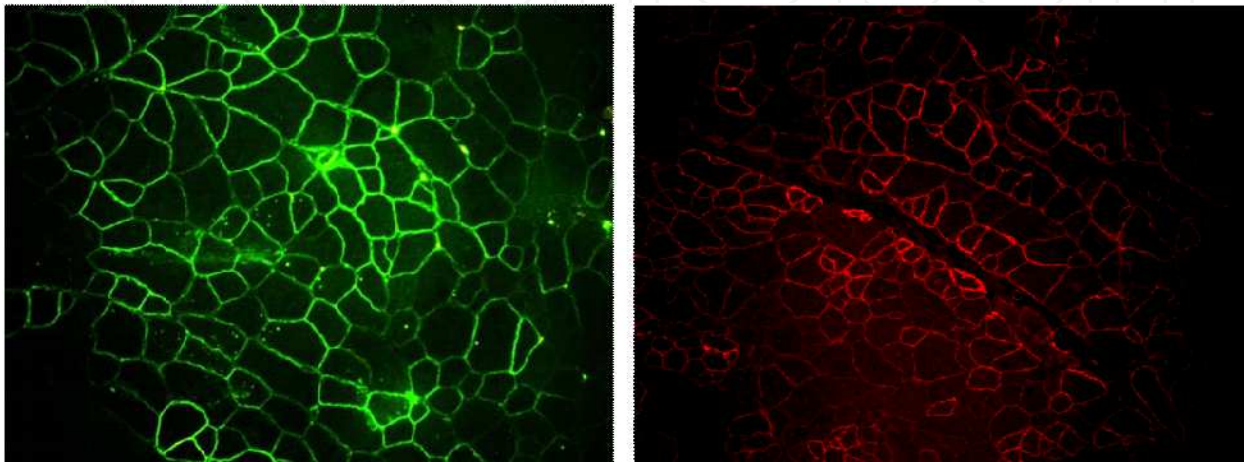
Laminopathies are caused by mutations in the LMNA gene, which encodes the inner nuclear envelope proteins lamin A and C, which interact with several proteins in the nucleus and inner nuclear membrane (Worman et al., 2000). Emery-Dreifuss muscular dystrophy (EDMD) has been identified due to a mutation in LMNA gene encoding A-type lamin, or in emerin gene encoding nuclear protein (Bione et al., 1994). Clinical symptoms are related to humero-peroneal weakness and dilated cardiomyopathy with conduction defects (Emery, 2000). Mutations in LMNA gene cause cardiomyocyte nuclear envelope abnormalities, leading to dilated cardiomyopathy in human patients (Gupta et al., 2010) and LGMD 1B (Muchir et al., 2000).

## **3. Pre-clinical & clinical approaches for DMD**

Several pre-clinical research regimes have displayed that DMD pathology can be improved by functional expression of mini-or micro-dystrophin gene variants (Deconinck et al., 1996).

### 3.1 Recombinant adeno-associated virus vector (rAAV) mediated gene therapy

Gene therapy for DMD aims to compensate for dystrophin loss-of-function by different gene transfer approaches. To prevent muscle degeneration, around 30% of normal levels of dystrophin protein is likely to be required (Neri et al., 2007). Transfer of recombinant genes encoding full-length (~11 kb), mini- (>5 kb) or micro- (<5 kb) dystrophin recombinant gene into DMD-affected muscle is one of the proposed therapies to induce dystrophin protein expression. A Phase I clinical trial has been completed in which a eukaryotic expression plasmid encoding full length human dystrophin was injected directly into the muscles of DMD patients (Fardeau et al., 2005).



The mdx mice treated with AAV-mediated microdystrophin gene transfer (left-hand panel), or PMO antisense-induced skipping of the mutant exon 23 (right-hand panel) showed dystrophin expression at the sarcolemma in the TA muscle of mdx mouse.

Fig. 2. Dystrophin immunofluorescence staining of muscle.

Dystrophin expression at low levels was shown in all patients without any safety concerns and notably without a detectable immune response to dystrophin. A Phase I/II study is currently in preparation to evaluate this non-viral plasmid-mediated gene therapy using vascular systemic routes of administration to target multiple muscle groups. However, even with the aid of more sophisticated delivery strategies such as electrotransfer or even with enhanced vector configurations such as minicircles and episomes, effective plasmid gene therapy for DMD still has major hurdles to overcome to reach the gene-transfer efficiencies required for effectiveness. Therefore a range of replication-defective viral vectors have been evaluated as attractive delivery systems to mediated dystrophin gene transfer and DMD gene therapy.

In particular, gene transfer into the nuclei of muscle fibres using vectors based on adeno-associated virus (AAV) is one of the most promising delivery approaches for the therapy of muscle disease. AAV is a single-stranded non-pathogenic virus, and derived vectors of various serotypes can efficiently transduce, not only by local muscle injection, but also, notably, by vascular systemic delivery to widespread muscle groups and the heart. The tropism of various AAV vector serotypes for differentiated post-mitotic muscle tissues can be very high, reaching >90%.

However, there is a hurdle to AAV-mediated DMD gene therapy, since the full dystrophin coding sequence spans 14 kb mRNA with an open reading frame of >11 kb, and the

packaging capacity of AAV virus vectors is generally thought to be <5 kb. Thus, on the basis of an understanding of the genotype-phenotype correlates in DMD and BMD, and based on structural knowledge of the dystrophin protein, recombinant dystrophin cDNAs have been engineered to produce, partially deleted, but highly functional microdystrophin products in both *mdx* mouse and *CXMD* dog models (Athanasopoulos et al., 2004, Foster et al., 2008, Koo et al., 2011). These genes can be packaged successfully inside AAV vector particles and can be delivered at high efficiency to skeletal muscles to rescue the dystrophin-deficient phenotype in animal models of DMD (**Figure 2**, left hand panel).

The life cycle of AAV vectors results in delayed expression, and it takes several weeks for full transcriptional activity to be established (Ferrari et al., 1996). Recently, self-complementary AAV (scAAV) vectors have been developed for fast expression by bypassing rate-limiting, second-strand DNA synthesis (McCarty 2008). However, scAAV can only retain smaller transgene cassettes compared to single stranded AAV (ssAAV). Moreover, high titres of rAAV are required for systemic delivery (Gregorevic et al., 2004).

AAV-mediated gene therapy has attractive characteristics and some advantages over other vector systems for several reasons. AAV vector particles are very stable and resistant to significant variation in pH and temperature. AAV vectors have an ability of long-term transgene expression without significant immune response. Therefore, it is a promising delivery vehicle for the transfer of therapeutic genes for the treatment of inherited diseases. Recombinant AAV-mediated gene therapy has been approved for use in over 40 clinical trials for various genetic diseases (Mueller et al., 2008). In order to generate recombinant AAV vector for the transfer of therapeutic genes, the genes for the capsid proteins and replication proteins are replaced with the gene of interest and packaged by ITRs. A Phase I clinical trial involving local intramuscular injection of AAV vectors to transfer a microdystrophin variant in DMD patients has been conducted by the groups of Mendel, Xiao and Samulski, in collaboration with Asklepios Biopharmaceuticals. However, microdystrophin expression was only detected in two of six patients treated at very low levels and two patients exhibited pre-existing dystrophin specific T cells (Mendell et al., 2010). Further work to enhance the expression and functionality of microdystrophins is still required due to reported failure of certain microdystrophin variants to protect muscle integrity in larger animal models (Sampaolesi et al., 2006).

### 3.2 Adenovirus-mediated gene therapy

Adenovirus is another vehicle which can be used to transfer dystrophin genes towards DMD mediated gene delivery applications. Due to the large gene capacity of the adenoviruses, large cDNA cassettes, up to 7 kb for E1/E3 deleted Ad vectors, can be transferred into the muscles. However, adenovirus is highly immunogenic and this may cause loss of transgene expression through immune responses. Moreover, the packaging capacity is still too small for the transfer of full length dystrophin (approximately 11 kb cDNA). To increase the packaging capacity of the viruses, helper dependent adenovirus vectors have been developed (Fisher et al., 1996). These vectors contain only ITR and capsid genes and so increase the packaging genome capacity of adenoviruses up to 36 kb; they are also less immunogenic compared to E1 gene-deleted adenovirus due to the lack of viral genes. Adenovirus-mediated full length dystrophin cDNAs have been successfully transferred to *mdx* mice leading to muscle improvement (Dudley et al., 2004). Cell transplantation of genetically corrected



mesenchymal cells (MSCs) by adenovirus carrying microdystrophin gene was attempted and showed successful dystrophin expression in MSCs in *mdx* mice (Xiong et al., 2007).

### 3.3 Modulation of exon-splicing patterns with antisense oligonucleotides

About 70% of DMD and BMD cases are caused by genomic deletions, leading to the loss of one or more exons (Aartsma-Rus et al., 2006). Frameshift deletions, which juxtapose out-of-phase exons in the dystrophin gene, cause complete loss of expression of dystrophin and a DMD phenotype, whereas juxtaposition of in-phase exons leads to the milder BMD. Inhibition of the splicing of specific exons, by so called exon skipping, using antisense oligonucleotides (AONs) can induce exclusion of targeted exons and skipping of frame-shift exons, leading to restoration of disrupted reading frames and expression of BMD-type dystrophin molecules (**Figure 2**, right hand panel). AONs are designed to hybridize to consensus exon recognition or exonic splicing enhancer (ESE) sequences on dystrophin pre-mRNA, and antisense-induced exon skipping is thought to occur by interfering with binding of serine/arginine-rich (SR) proteins which play crucial roles in recruiting the splicing machinery (**Figure 3**).

AON-induced exon skipping to restore functional but truncated dystrophin protein expression has previously been demonstrated in animal models of DMD both *in vitro* (Graham et al., 2004) and *in vivo* (Yokota et al., 2009), and in DMD patient cells *in vitro* in culture (van Deutekom et al., 2001), and in DMD muscle explants (Arechavala-Gomez et al., 2007). On the basis of these pre-clinical studies, a number of patient trials, phase I and more recently phase 2, have been undertaken. In the first of these, four DMD patients carrying appropriate deletions received a single intramuscular injection of a high dose of an AON with a 2'-O-methylphosphorothioate backbone (PRO051), which targets exon 51. Each patient showed specific exon 51 skipping, myofibre expression of dystrophin protein, which was detectable at 3 to 12% of normal levels four weeks after injection. No clinically adverse events were detected (van Deutekom et al., 2007). In the second trial, the AON AVI-4658, which has a phosphorodiamidate morpholino (PMO) backbone and targets a slightly different intraexonic sequence (+68+95) of exon 51, has been injected intramuscularly in a dose-escalating trial into nine DMD boys. At the higher doses, this PMO AON produced good levels of local dystrophin protein production in treated muscles; the intensity of dystrophin staining was up to 42% of that seen in healthy muscle. The treatment had no adverse effects (Kinali et al., 2009). The clinical evaluation has been extended to 12 week systemic delivery of both exon 51 AONs and results have very recently been reported. Both chemistries showed no adverse effects and dose-dependent restoration of dystrophin production was clearly seen; functionality of this expressed dystrophin protein was established by the detection of other dystrophin-associated proteins at the sarcolemma (for AVI-4658), and by a modest but not statistically significant improvement in the patient six minute walk test after 12 weeks of extended treatment (for PRO051) (Goemans et al., 2011). On the basis of results seen in the *mdx* model using various dosing regimen over extended periods (Malerba et al., 2011), further clinical studies are required.

PMOs and 2'OMe AONs both have excellent safety profiles (van Deutekom et al., 2007), but PMOs have certain advantages over 2'OMe AONs. They give more sustained, consistent exon skipping in the animal *mdx* model *in vivo* (Heemskerk et al., 2009), and in human muscle explants (Arechavala-Gomez et al., 2007). PMOs can be conjugated to cell-penetrating

peptides (PPMO) that improve their deliverability and hence efficacy dramatically (Moulton et al., 2007, Yin et al., 2010).

The drug company AVI BioPharma has performed preclinical studies with AVI-5038 in collaboration with the charity Charley's fund. AVI-5038 is a PPMO targeted to skip exon 50 of the dystrophin gene. Repeated weekly intravenous bolus injection over four weeks at a low dose of this conjugated PMO was shown to be well-tolerated; however higher doses administered weekly for 12 weeks showed significant toxicological effects, particularly in relation to the kidney. As yet this problem has not been resolved, and an unconjugated version of the same PMO (AVI-4038) is being developed for clinical trial. There are a number of alternative peptide conjugates that show promise as enhancers of deliverability and are undergoing rapid pre-clinical development (Yin et al., 2010). The next planned UK phase I trial by the MDEX consortium will involve conjugation of a PPMO developed for the targeted skipping of exon 53 (Popplewell et al., 2010) and is supported by a Wellcome Trust. The Dutch are currently performing a phase I trial using a 2'OMe PS AON for the targeted skipping of exon 45. However, it should be noted that only 8%, 4%, 13% or 18% of DMD patient mutations should be convertible into a BMD phenotype by a single AON exon 45, 50, 51 or 53 skipping, respectively. Personalized molecular medicine for each skippable DMD deletion is necessary and this would require the optimization and clinical trial workup of many specific AONs. It has been suggested that multi-exon skipping, using cocktails of AONs or chemically linked AONs, around deletion hotspots (eg exons 45-55) may have the potential to treat approximately 65% of DMD patients (Adams et al., 2007). Such a strategy has been shown to work in mdx mice, but this has not yet been achieved in DMD patient cells.

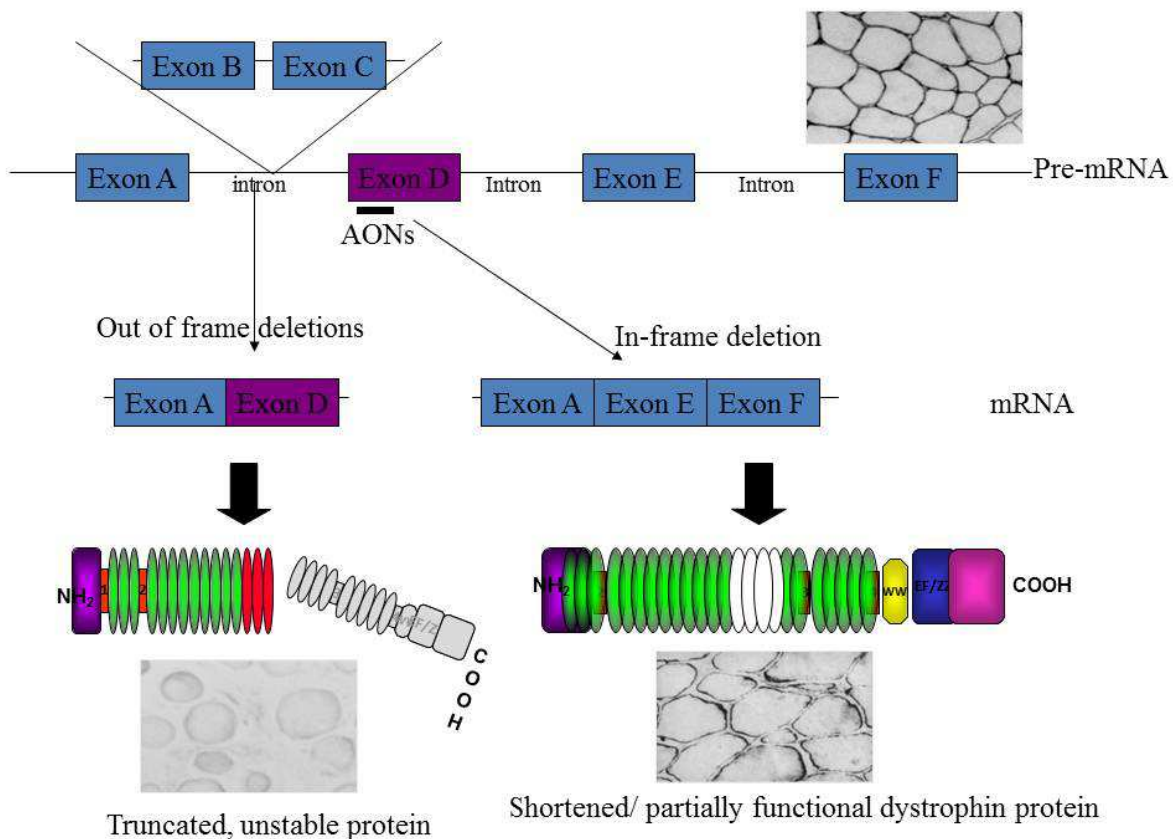


Fig. 3. AONs mediated exon skipping for Duchenne muscular dystrophy.

This gene therapy restores the open reading frame of the disrupted reading frame to allow synthesis of shortened dystrophin. The in-frame transcript contains both the N- and C-terminal domains of dystrophin and is partially functional since these domains have important signalling functions between the extracellular matrix and cytoskeleton. The in-frame transcript of dystrophin produced by AONs exon skipping ameliorates the severe muscle damage seen in *mdx* mice.

There are further obstacles to be overcome for AON-induced exon skipping to be a viable gene therapy for DMD. The cost implications may end up being prohibitive for many patients; since AONs are rapidly cleared from the circulation, regular administrations of high doses of AON would be required for therapeutic effect. Secondly, although deliverability, particularly to the heart, is enhanced with the use of conjugated PMOs, their potential toxicological and immunogenic problems need to be addressed. Lastly, the need for personalized medicine will require the completion of many expensive, lengthy clinical trials of many AONs.

### 3.4 Cell transplantation therapy

Cell-based gene therapy is another promising approach to treat DMD. Transplantation of genetically modified adult muscle stem cells or healthy wild-type donor cells has great potential to regenerate skeletal muscle cell tissues. For stem cell therapy, adult muscle stem cells, including mesoangioblasts, MDSCs (muscle derived stem cells), adult progenitor cells, AC133+ cells, bone marrow-derived stem cells and side population (SP) cells can be isolated from either muscle biopsies or blood (Farini et al., 2009). Isolated stem cells can be transduced *in vitro* by retroviral or lentiviral vectors to permanently introduce the micro- or mini-dystrophin gene. Retrovirus or lentiviral genome can integrate into the infected cell genome subsequent to cell divisions. Therefore this is a promising vehicle for the correction of muscle satellite stem cells. These genetically modified cells or healthy wild-type donor cells are expanded *ex vivo* and subsequently injected systemically to traffic and target to muscles. If taken from the patient themselves, transplantation of autologous gene-modified stem cells has the advantage to potentially avoid adverse immune responses and cell rejection.

Intramuscular myoblast transplantation in DMD yields dystrophin-positive myofibres at levels of 20-30% up to 18 months after transplantation (Skuk et al., 2007). However, intramuscular myoblasts transfer faces potential limitations in terms of cell migration, lack of systemic whole-body delivery and poor cell survival. There are currently two approaches using a cell transfer platform for systemic delivery of regenerative cell transplants to skeletal musculature, namely the mesoangioblast and the CD133+ stem cell systems. Following lentiviral vector-mediated gene transfer, dystrophin-expressing fibres have been shown in dystrophic dogs after transplantation of genetically modified mesoangioblasts which are vessel-associated progenitor cells. In this study, transplantation efficiency seemed higher in recipients treated with wild-type donor mesoangioblasts compared with autologous gene-modified mesoangioblasts (Sampaolesi et al., 2006), but uncontrolled effects of immune suppression regimes may complicate the interpretation. Even so, a clinical trial of mesoangioblast cells therapy for DMD using tissue -matched wild-type donor cells has been tried (Cossu et al., 2007). CD133+ stem cell therapy is another promising approach introduced recently, making systemic delivery and correction possible. CD133+ cells can be

isolated from peripheral blood or skeletal muscle tissue, and differentiated into muscle, hematopoietic and endothelial cell lineages (Peault et al., 2007). A Phase I clinical trial of autologous transplantation of CD133+ stem cells in DMD boys has shown increased potential to contribute to muscle repair without adverse effects (Torrente et al., 2007). However, interaction between satellite cells and stem cells still remains largely unknown to date. Detailed understanding of the biological mechanism of interaction and structure/function relationship between muscle and the multiple components of these therapies can aid the search for an effective cure for DMD and other muscle diseases in the immediate future.

### **3.5 Muscle augmentation**

Neuromuscular disorders, sarcopenia, cancer, AIDS and general insufficient energy intake can decrease the muscle mass with consequent irreversible loss of body weight (Matsakas et al., 2009). Several genetic based strategies have been tried to manipulate expression of specific molecules involved in muscle growth to counteract this muscle loss. One of the most promising approaches is based on the inactivation of the biological activity of myostatin or its receptor, Activin type 2b (ActRIIb). This strategy has a therapeutic potential to treat DMD by inhibiting the severe muscle loss.

#### **3.5.1 Inactivation of Myostatin**

Myostatin (named also GDF-8) is a member of transforming growth factor  $\beta$  superfamily of growth factors. Myostatin is initially synthesized as inactive pre-propeptide and it is cleaved to produce a smaller peptide (Thies et al., 2001, Hill et al., 2002) that is biologically active only after further post-translational processing including the formation of a homodimeric protein. Some animals carrying different types of mutations that inactivate the functionality of myostatin, present a significant increase in muscle mass (Tobin et al., 2005). In myostatin mutant mice, individual muscles weigh twice as much compared to wild type mice (McPherron et al., 1997) due to hyperplasia and hypertrophy. Other identified mutations of myostatin gene have led to new breeds of cattle (e.g. Belgian blue or Piedmontese (McPherron et al., 1997) and dogs (Mosher et al., 2007). These findings have made myostatin the first target of a genetic approach to silence the gene expression and induce increase in muscle mass as therapeutic treatment for muscle wasting diseases. Indeed, even if the biodistribution of myostatin has not been conclusively clarified, the fact that skeletal muscle is the tissue with the highest expression of myostatin (Sharma et al., 1999, McPherron et al., 1997) means this protein is an appealing target for muscle augmentation strategies. Several strategies aiming to knock down myostatin to induce muscle mass augmentation have been preclinically tested: adeno-associated viral vectors have been used to deliver myostatin propeptide and so inactivate the growth factor by making it unavailable for binding its receptor (Foster et al., 2009; Matsakas et al., 2009). Follistatin-related gene (FLRG) and growth and differentiation factor association protein (GASP-1) are both able to bind to the homodimeric protein myostatin and inhibit its functionality even if the effect is less pronounced compared to the use of a pro-peptide coded by viral vectors (Thies et al., 2001, Hill et al., 2002). Other strategies to inactivate myostatin are the use of antibodies raised against this growth factor in order to make it unavailable to the receptor. The antibodies JA16 or MYO-029 have been preclinically tested with the latter being also used for a clinical trial in humans where it demonstrated good tolerability and safety (Hill et al., 2002). A

recent strategy to knock down myostatin is based on the use of antisense oligonucleotides to bind the pre-mRNA, skip the second exon of myostatin and induce the formation of an out of frame transcript unable of being translated in a functional protein (Kang et al., 2011). The systemic administration of antisense oligonucleotide in mouse showed encouraging results even if the effect was less pronounced compared to the overexpression of pro-peptide or the myostatin specific antibody administration.

### 3.5.2 Inactivation of Activin receptor IIb

A second target used to significantly decrease the biological effect of myostatin is to inactivate its binding to the receptor, activin receptor IIb (ActRIIb). The overexpression of myostatin antagonist follistatin, which binds ActRIIb sterically, prevents the binding of myostatin (Rodino-Klapac et al., 2009) and induces an increase in muscle mass. Another study explored the possibility of inhibiting myostatin by using RNAi against ActRIIb and restore quasi dystrophin by AAV-U7 mediated exon skipping in a mouse model of muscular dystrophy (Dumonceaux et al., 2010). Recently in the same animal model the use of soluble ligands of ActRIIb as peptides, including the extracellular portion of the ActRIIb fused to the Fc portion of murine IgG (sActRIIb), has been shown to improve skeletal muscle mass and functional strength (Pistilli et al., 2011).

### 3.6 Drug-induced read-through of nonsense mutations

Although most DMD cases are caused by large intragenic deletions, 10-15% of DMD mutations are nonsense point mutations which cause premature termination codons (PTCs) in the dystrophin mRNA. PTC 124 is a drug designed to induce translational read through of PTCs, thus inducing expression of dystrophin and reducing nonsense-mediated mRNA decay. Because of the mechanistically structural differences between PTCs and natural stop codons, PTC 124 appears to preferentially recognize mutant nonsense codons without interfering strongly with native termination codons of unrelated genes. PTC 124 has been proven to enhance dystrophin expression in both primary DMD muscle cells and animal models (Welch et al., 2007). A Phase 2b clinical trial in subjects with nonsense-mutation-mediated DMD/BMD has been completed. Unfortunately, PTC 124 did not render any improvement in the six minute walk distance.

## 4. Conclusion

Preclinical and clinical genetic and cell-based therapy trials are currently progressing rapidly, with the interaction of multiple research units, biotechnology companies and patient groups. Several studies in the clinic are now reporting promising results with restoration of dystrophin expression in local muscle fibres. Future perspectives of the current strategies will be to overcome obstacles in the way of them becoming therapeutic treatments for DMD

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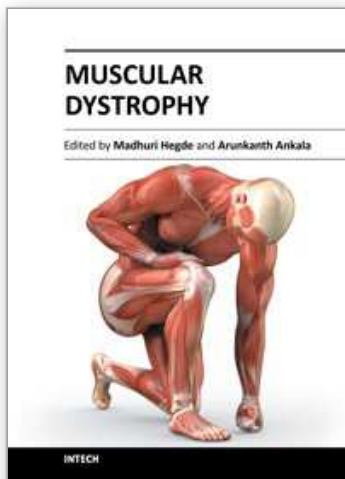
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## **Muscular Dystrophy**

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With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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