

Exon Skipping and Myoblast Transplantation: Single or Combined Potential Options for Treatment of Duchenne Muscular Dystrophy

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1. Introduction

Edward Meryon, an English doctor, described Duchenne muscular dystrophy (DMD) for the first time, but the symptoms and the histology typical of this condition were firstly described by Duchenne de Boulogne in 1861. The dystrophin gene (*dys*) was firstly identified by Kunkel and coworkers (Kunkel et al., 1986), while Hoffman and colleagues (Hoffman et al., 1987) identified the gene product dystrophin. This protein is lacking in DMD patients' muscles and, due to its essentiality in membrane stability, its absence induces contraction-related membrane damage and the activation of the inflammatory cascade, leading to muscle failure, necrosis, and fibrosis (Hoffmann and Dressman, 2001; Blake et al., 2002; Palmieri B. and Sblendorio V., 2006). This condition affects primarily human and animal skeletal and cardiac muscle and it is defined as an X-linked recessive disease with the most cases inherited from carrier mothers, and about a third of cases occurring as *de novo* mutations in the infants. DMD is present at birth, but clinical symptoms are not evident until 3 to 5 years of age (leg weakness, increasing spine kyphosis, and a waddle-like gait) and usually its diagnosis is made on the basis of gait spine abnormalities from 4 to 5 years after birth (Dubowitz et al., 1975; Jennekens et al., 1991). DMD patients display problems in climbing stairs and rising up from the floor; they are unable to run, and in a variable way, most of them lose ambulation by 7 to 12 years (Iannitti et al., 2010). Moreover, other characteristics of this

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disease are the progressive loss of respiratory function that can lead to respiratory failure, scoliosis, weight loss, cardiomyopathy, and finally death, as a result of respiratory and cardiac complications (Iannitti et al., 2010). The continuous muscle wasting, that characterizes this pathological condition, puts DMD patients, from 8 to 12 years of age, in wheelchairs with scoliosis developing in 90% of boys who use a wheelchair full-time and die in the late teens or early twenties due to respiratory/cardiac failure after a worsening of symptoms (Emery, 1993). In children with DMD, non-progressive abnormalities of the central nervous system have also been observed. In fact, the mean intelligence quotient of these patients is 82 which is 18 points under the mean value of the healthy population, while 30% of the patients have a quotient under 75 (Bresolin et al., 1994). In particular, verbal intelligence is primarily affected and 80% of DMD patients display atypical electroretinography with the most prominent portion of the normal electroretinogram, the wave b, that is absent (Billard et al., 1992; Sigesmund et al., 1994).

Inflammation, mediated by neutrophils, macrophages and cytokines, also seems to be involved in the damage of dystrophic muscles (Whitehead et al., 2006). Gosselin and colleagues (Gosselin et al., 2004) described the important role played by inflammation showing that a persistent inflammatory response has been observed in dystrophic skeletal muscle leading to an alteration in extracellular environment; it includes an increase in inflammatory cells, such as macrophages and elevated levels of various inflammatory cytokines like tumor necrosis factor alpha (TNF- α) that contributes to muscle degeneration, while pro-fibrotic cytokines, such as transforming growth factor beta (TGF- β), can account for a progressive fibrosis. Experimental studies, using the DMD mdx mouse model, support this fact reporting that the depletion of inflammatory cells, such as neutrophils, cromolyn blockade of mast cell degranulation or pharmacological blockade of TNF, reduces necrosis of dystrophic myofibers (DeSilva et al., 1987).

2. Dystrophin

The human dystrophin gene (13.973 nucleotides), dys, maps at the Xp21.1 locus; it is encoded by a 2.25-Mbp gene with 79 exons and 99.4% of its sequence is composed of introns (the fully processed transcript is only 14 Kbp) (Kunkel et al., 1986). The DMD gene can produce different dystrophin isoforms through alternative promoter usage and splicing of pre-mRNA and the predominant isoform is an approximately 427-kDa cytoskeletal protein that consists of 3685 amino acids constituting 5% of sarcolemmal protein and 0.002% of total striated muscle protein (Hoffman et al., 1987; Koenig et al., 1988).

Four domains constitute the structure of full length dystrophin, i.e. an N-terminal "acting binding" domain, a middle "rod" domain consisting of spectrin-like repeats, a cysteine-rich domain encoded by exons 62 to 70 and a C-terminal domain. The last two domains play a key role in the assembly of the dystroglycan complex and in the sarcolemmal function (Petrof, 2002; Palmieri and Sblendorio, 2006). When a mutation and deletion occur in the dystrophin gene, as observed in DMD patients, the protein cannot be produced leading to its complete absence in muscle fibers. Dystrophin belongs to a group of proteins called dystrophin glycoprotein complex (DGC), which also include cytoskeletal actin, the dystroglycan integral membrane proteins, the syntrophins, dystrobrevins and α -catulin (Brown et al., 1997). Dystrophin links the actin intracellular microfilament network to the extracellular matrix and its absence changes the level and localization of DGC, making the sarcolemma fragile and

muscle fibers prone to degeneration during repeated cycles of muscle contraction and relaxation. Actin associates with the N-terminal of dystrophin, in a region displaying two calponin homology domains (Corrado et al., 1994; Norwood et al., 2000; Way et al., 1992), while the other proteins bind to the C-terminal region of dystrophin. The association of b-dystroglycan with dystrophin is mediated by a cysteine-rich region of dystrophin that contains a protein module with two highly conserved tryptophans (WW domain) and 2 EF-handlike motifs (Huang et al., 2000; Jung et al., 1995). Furthermore, b-dystroglycan associates with the extracellular protein a-dystroglycan which, in turn, connects to laminin in the extracellular matrix (ECM) (Henry et al., 1998; Henry et al., 1999; Hohenester et al., 1999). Dystrophin, through its association with actin and dystroglycan, represents a key bridge between the ECM and cytoskeleton, playing an important role in the structural integrity of the muscle cell membrane. The absence or disruption of dystrophin, observed in DMD, also exerts some effects on the central nervous system (CNS) function. In fact the dystrophin role in the positioning of receptors and channels is relevant at the synapse level where the neuromuscular junction exerts an important role on the synapse structure and function (Hall et al., 1993; Sanes et al., 1999). Dystrophin is localized in the deep regions of junctional folds at the post-synaptic face (Bewick et al., 1992; Sealock et al., 1991) and, through its association with its complex of proteins (Fig. 1), dystrophin plays both structural and signalling roles.

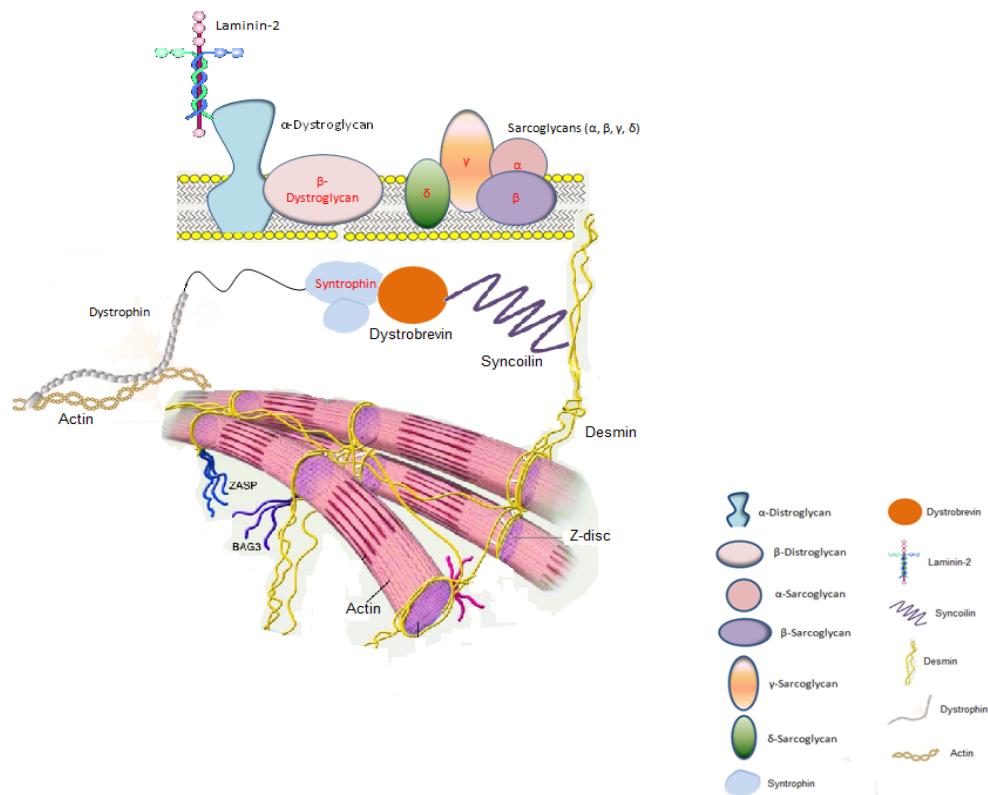


Fig. 1. Complex of proteins associated with dystrophin.

Other clinical aspects of the DMD pathophysiology have been reported. These are cognitive impairment and lower intelligence quotient (IQ) (average = 85) observed in boys with DMD, disordered CNS architecture, abnormalities in dendrites and loss of neurons. Moreover, at the biochemical level, the bioenergetics of the CNS is abnormal, and there is an increase in the concentration of choline-containing compounds, indicative of CNS pathology.

Dystrophin expression is regulated by seven independent promoters, three of which regulate the expression of full-length isoforms, while four intragenic promoters regulate the expression of different short isoforms in various tissues. Two additional isoforms are considered to be full-length and are expressed in the brain. The mutation that affects dystrophin promoter regions and regulates the expression of brain isoforms, may be the cause of neurological symptoms in some patients with DMD. Each of the additional transcripts results in the expression of multiple dystrophin proteins (Dp) that are indicated according to their molecular weight: Dp427 muscle, Dp427 brain, Dp427 purkinje, Dp260, Dp140, Dp116, and Dp71 (Muntoni et al., 2003). These last four variants contain unique first exons and lack the actin binding domain, suggesting that they may have functions that are different from the ones ascribed to full-length dystrophin. Dp260 in the retina and Dp71 in the brain and other tissues restore the integrity of DGC, but only Dp260, the longest of the short isoforms, restores some functional integrity in the dystrophic muscle. These findings suggest that a better bridge between ECM and actin is more necessary for the improvement of dystrophic muscle function than the one provided by the Dp71 variant.

Alternative splicing of exons 71 to 74 and 78 increases the diversity of transcripts; the first splicing regulates interactions with syntrophins. These exons can be spliced singularly or in a different combination, generating a series of in-frame spliced variants.

The elimination of exons 73 and 74 in any of these transcripts generates a functional protein that lacks the syntrophin binding sites (Yang et al., 1995; Newey et al., 2000). The splicing of exons gives a translational frame shift, producing the substitution of the last 13 amino acids of the predominantly hydrophilic C-terminal region with 31 hydrophobic amino acids. This process is regulated in a developmental and tissue-specific way (Tennyson et al., 1996). The hydrophobic splice variant is abundant in the cerebral cortex and retina and it serves to regulate the binding of dystrophin to α -catulin and its associated proteins (Roberts et al., 1998).

3. Searching criteria and aim

We have been searching Pubmed/Medline, using the key words "Duchenne", "Muscular" and "Dystrophy" combined with "Exon", "Skipping", "Immunosuppressant", "Stem", "Cells", "Myoblast" and "Transplantation", in order to collect and analyze all the recent advances in DMD, focusing on clinical trials performed in humans. This chapter highlights the most promising therapeutical approaches to DMD, i.e. exon skipping and myoblast transplantation with some details about the immunosuppressive therapy.

4. Immunosuppressant drugs

Among the scientific community there is a growing interest in the use of immunosuppressant drugs that may potentially give clinical benefits during the DMD course. The interest is due to the host transplant, potential immunosuppressant schedule

that should be suitable to increase myoblast or mesangioblast graft survival supporting, in the meantime, the autologous crippled mass function. Among immunosuppressant drugs, corticosteroids slow DMD progression and, in particular, two corticosteroids, i.e. prednisone and deflazacort have been extensively used because of their ability to improve skeletal muscle function. Research has also focused on the use of suppressing drugs acting against TNF level and suppressing calcineurin signals.

The long-term effects of deflazacort treatment has been investigated according to two treatment protocols from Naples (N) and Toronto (T) in boys, aged between 8 and 15 years, who were affected by DMD and had 4 or more years of deflazacort treatment (Biggar et al., 2001). Thirty seven boys were treated with protocol N, using deflazacort at a dose of 0.6 mg/kg per day for the first 20 days of the month and no deflazacort for the remainder of the month. Vitamin D and calcium were administered daily to boys with osteoporosis. Deflazacort treatment started between 4 and 8 years of age. Thirty two were treated with protocol T, using deflazacort at a dose of 0.9 mg/kg per day, plus vitamin D and calcium daily. Treatment started between 6 and 8 years of age. All boys were monitored every 4 to 6 months and the results were compared with age-matched control subjects in the two groups (19 for protocol N and 30 for protocol T). It was observed that: 1) for the boys treated with protocol N, 97% were ambulatory at 9 years (control, 22%), 35% at 12 years (control, 0%) and 25% at 15 years (control, 0%); 2) for the 32 boys treated with protocol T, 100% were ambulatory at 9 years (control, 48%), 83% at 12 years (control, 0%) and 77% at 15 years (control, 0%); 3) in boys aged 13 and older, scoliosis developed in 30% of boys in protocol N, 16% in protocol T, and 90% of control subjects. 30% of boys, who were treated according to protocol T, had asymptomatic cataracts, but they did not require any treatment. Fractures occurred in 19% of boys in protocol N (controls: 16%) and 16% of boys in protocol T (controls: 20%). Summarizing, long term deflazacort treatment has beneficial effects on both protocols, although protocol T seems to be more effective and it is frequently associated with asymptomatic cataracts.

The same group (Biggar et al., 2006) designed a study involving 54 boys (30 treated with deflazacort), aged between 7 and 15 years, affected by DMD, who were reviewed retrospectively. The boys, untreated with deflazacort, stopped walking at 9.8 ± 1.8 years, while 7 out of 30 treated boys stopped walking at 12.3 ± 2.7 years ($P < 0.05$). Among the 23 boys who were still walking, 21 were 10 year older; pulmonary function was significantly greater in 15 year old treated boys ($88\% \pm 18\%$) than in untreated boys ($39\% \pm 20\%$) ($P < 0.001$). Between 9 and 15 years, the treated boys were shorter and, between 9 and 13 years, the treated boys weighed less. After 13 years, the treated boys maintained their weight, whereas the untreated boys lost weight. Asymptomatic cataracts developed in 10 out of 30 boys who had received deflazacort.

Another clinical study compared the course of 74 boys, aged from 10 to 18 years, and affected by DMD, treated ($n = 40$) and untreated ($n = 34$) with deflazacort (Biggar et al., 2006). The treated boys were able to rise from a supine condition to standing, climb stairs, and walk 10 m without aids from 3 to 5 years longer than the untreated boys. After 10 years of age, the treated boys had a significantly better pulmonary function than the untreated boys and, after 15 years of age, 8 out of 17 untreated boys required nocturnal ventilation unlike the 40 treated boys. As for boys older than 15 years of age, 11 out of 17 untreated boys required assistance with feeding unlike the treated boys. Towards 18 years, 30 out of 34

untreated boys had a spinal curve greater than 20° if compared with 4 out of the 40 treated boys. By 18 years of age, 7 out of 34 untreated boys had lost 25% or more of their body weight (treated 0 out of 40) and four of those 7 boys required a gastric feeding tube. By 18 years of age, 20 out of 34 untreated boys had cardiac left ventricular ejection fractions, less than 45% if compared with 4 out of the 40 treated boys and 12 out of 34 died in their second decade (17.6 ± 1.7 years), primarily of cardiorespiratory complications. Two out of 40 boys, treated with deflazacort, died at 13 and 18 years of age from cardiac failure. The treated boys were significantly shorter, did not have excessive weight gain, and 22 out of 40 had asymptomatic cataracts. Long bone fractures occurred in 25% of boys in both the treated and untreated groups. The authors conclude that these long-term observations are the most encouraging. The major benefits of daily deflazacort appear to be the prolonged ambulation, improvement in cardiac and pulmonary functions, delay in the need for spinal instrumentation, and a greater independence for self-feeding. According to the described last two studies, deflazacort has a significant impact on health, quality of life, and healthcare costs for boys with DMD and their families and it is associated with a few side effects.

Houde and coworkers (Houde et al., 2008) collected data over an 8-year period for 79 patients with DMD, 37 of whom were treated with deflazacort. Deflazacort (dose of 0.9 mg/kg adjusted to a maximum of 1 mg/kg according to the side effects) was started when the boys showed a functional decline resulting in ambulating difficulties. The mean length of treatment was 66 months.

The treated boys stopped walking at 11.5 ± 1.9 years, whereas the untreated boys stopped walking at 9.6 ± 1.4 years. The cardiac function, assessed by echocardiography every 6 to 12 months, was better preserved as shown by a normal shortening fraction in treated ($30.8\% \pm 4.5\%$) versus untreated boys ($26.6\% \pm 5.7\%$, $P < 0.05$), a higher ejection fraction ($52.9\% \pm 6.3\%$ treated versus $46\% \pm 10\%$ untreated), and lower frequency of dilated cardiomyopathy (32% treated versus 58% untreated). No change was observed in blood pressure, left ventricle end-diastolic diameter, or cardiac mass. Scoliosis was much less severe in treated ($14^\circ \pm 22.5^\circ$) than in untreated boys ($46^\circ \pm 224^\circ$) and no spinal surgery was necessary in treated boys. Limb fractures occurred in 24% of treated and in 26% of untreated boys, whereas vertebral fractures occurred in the treated group only (7 out of 37 compared with zero in the untreated group). In both groups, weight excess was observed at 8 years of age, and its frequency tripled between the ages of 8 and 12 years. More patients had weight excess in the treated group (13 out of 21 [62%]) than in the untreated group (6 out of 11 [55%]), at 12 years of age. Cataracts developed in 49% of treated patients and, in almost all of these patients, they developed after at least 5 year treatment. This study underlines that deflazacort use in DMD prolongs walking for at least 2 years, slows the decline of vital capacity, and postpones the need for mechanical ventilation. The quality of life seems to improve in terms of prolonged independence in transfers and rolling over in bed, as well as sitting comfortably without having to resort to surgery.

A study determined and compared the long-term effects of prednisone and deflazacort on 49 boys, aged between 12 and 15 years, with DMD over a 7-year follow up period (Balaban et al., 2005). Eighteen boys were treated with prednisone, 12 with deflazacort, and 19 had no drug treatment. Analyzing their lower and upper limb motor functions, pulmonary function, prevalence of surgery for scoliosis and side effects, they reached these results: the

boys in the steroid groups were significantly more functional and performed better on all tests than the untreated boys ($P < 0.05$); there was no significant difference between deflazacort- and prednisone-treated groups ($P > 0.05$); the number of boys having scoliosis surgery among the treated groups was significantly less than the one of untreated boys ($P < 0.05$); the control group's capacity had decreased and was significantly less than the one of both the prednisone and deflazacort treated boys; both deflazacort and prednisone had beneficial effects on the pulmonary function and scoliosis; cataracts, hypertension, behavioural changes, excessive weight gain, and vertebral fracture were noted as serious side effects. The results of this long-term study are very encouraging and both prednisone and deflazacort seem to have a significant beneficial effect on slowing the disease progress. Their use in DMD may prolong ambulation and upper limb function with similar potency. Both steroids are also able to improve pulmonary function, more than delay the need for spinal interventions, with similar therapeutic profiles.

A study was performed in 17 patients affected by DMD, aged between 17 and 22 years, treated with deflazacort (0.9 mg/kg/day) and compared with DMD patients who did not receive any treatment, in order to evaluate the involvement of cardiac and sternocleidomastoid muscles by means of magnetic resonance imaging (MRI) measurement of T2 relaxation time and the left ventricular systolic function (Mavrogeni et al., 2009). This study showed that DMD patients, treated with deflazacort, present a better cardiac and skeletal profile compared to DMD patients without medication ($p < 0.001$).

Dubowitz and colleagues (Dubowitz et al., 2002) reported a 5-year follow up of two 4-year-old boys, with classic DMD with an out-of-frame deletion in the Duchenne gene and absence of dystrophin in their muscle, who had a quite remarkable response to an intermittent, low dosage regime of prednisolone (0.75 mg/kg per day for 10 days each month or alternating 10 days on and 10 days off). In the first case, there was a complete remission of all clinical signs of dystrophy, sustained, almost fully, up to the present time; in the second case, the initial response was almost as marked, sustained for almost 5 years before showing a fairly rapid decline over the ensuing year that resulted in loss of independent ambulation at the age of 10. Both boys remained around the 50th percentile as for height and weight and showed no evidence of demineralization of bone on consecutive dual x-ray absorptiometry scanning of the spine nor any signs of chronic prednisolone toxicity. Although this study involved a limited number of patients, it showed that there might be an optimal window for treatment in the early stages of the disease and further larger-scale controlled studies should be targeted more selectively at this stage of the disease. This report also showed that a regime of low-dosage, intermittent prednisolone, with cycles of 10 day treatment, either per month or alternating with 10 days off treatment, is well tolerated in children affected by DMD.

Markham and colleagues (Markham et al., 2005) studied the effect of steroids in the cardiac function of patients with DMD. They evaluated the left ventricular systolic function and cardiac geometry of those subjects through a transthoracic echocardiogram; 111 patients, aged 21 years or younger, affected by DMD, were selected. They were divided into two groups: untreated (never exposed or treated for less than 6 months) and steroid-treated (steroids were administered longer than 6 months); the subjects did not differ in age, height, weight, body mass index, systolic and diastolic blood pressure, or left ventricular mass. Among the treated patients, 29 received prednisone and 19 received deflazacort. This study

showed that treatment, either with prednisone or deflazacort, appears to have an impact on the decline in cardiac function seen with DMD. The shortening fraction was significantly lower in the untreated group than in the steroid-treated one. The authors concluded that deflazacort and prednisone were equally effective in preserving the cardiac function. This study shows that the progressive decline in cardiac muscle function can be altered by steroid treatment. In particular steroid treatment brings a clinical improvement in respiratory and cardiac function in DMD patients, during and beyond their treatment period. Moreover, it has the potential to prolong their survival.

A randomized controlled trial of prednisone and azathioprine, involving 99 boys aged between 5 and 15 years and affected by DMD, was conducted with the aim to assess the longer-term effects of prednisone and to determine whether azathioprine, alone or in combination with prednisone, is able to improve strength (Griggs et al., 1993). The patients were divided into 3 groups: placebo; 0.3 mg/kg prednisone per day; 0.75 mg/kg prednisone per day. After 6 months, 2 to 2.5 mg/kg azathioprine per day was added to the first two groups and placebo added to the third group. The study showed that the beneficial effect of prednisone (0.75mg/kg per day) is maintained for at least 18 months and it is associated with a 36% increase in muscle mass. Weight gain, growth retardation, and other side effects were associated with prednisone and azathioprine did not have any beneficial effect. The authors conclude that prednisone beneficial effect is not the result of immunosuppression.

Kirschner et al. (Kirschner et al., 2008) conducted a randomized, multicenter, double-blind placebo-controlled trial. One hundred and fifty three patients were randomized to receive either placebo or 4 mg/kg ciclosporin A (CsA). After 3 months, both groups received additional treatment with intermittent prednisone (0.75 mg/kg, 10 days on/10 days off) for 12 months more. In each group, 73 patients were available for intention to treat analysis. Baseline characteristics were comparable in both groups. There was no significant difference between the two groups concerning primary (manual muscle strength according to the Medical Research Council) and secondary (myometry, loss of ambulation, side effects) outcome measures. Peak CsA values were measured blindly and ranged from 12 to 658 ng/mL (mean, 210 ng/mL) in the verum group. According to this study CsA does not improve muscle strength as a monotherapy and the efficacy of intermittent prednisone in DMD. Calcineurin inhibitors induced chronic nephrotoxicity as reported in a previous study (Naesens et al., 2009).

Sharma and coworkers (Sharma et al., 1993) tested CsA in 15 patients affected by DMD and observed an increase in the muscular force generation, measuring the titanic force and maximum voluntary contraction (MVC) of both anterior tibial muscles. Normally the titanic force and MVC declined during 4 months in patients with DMD. During 8 week CsA treatment (5 mg/kg per day), the titanic force significantly increased (25.8% 6 6.6%) and MVC (13.6% 6 4.0%) occurred in two weeks' time. The CsA side effects, gastrointestinal and flu-like symptoms were transient and self-limiting. Straathof and colleagues (Straathof et al., 2009) retrospectively analyzed 35 DMD patients' data, who were treated with 0.75 mg/kg prednisone per day intermittently, 10 days on/10 days off. Prednisone was started during the ambulant phase at the age 3.5 up to 9.7 years. The median period of treatment was 27 months. The authors reported the following results: the median age at which ambulation was lost was 10.8 years; 9 patients (26%) had excessive weight gain; 8 boys (21%) had a bone fracture that happened when four of those 8 children lost the ability to walk. Treatment was

stopped in 2 obese patients, 2 hyperactive boys, and 1 patient after a fracture. Based on the previously described data, the authors conclude that prednisone, 10 days on/10 days off, has relatively few side effects and extends the ambulant phase for 1 year if compared to historical controls.

5. Exon skipping

DMD is caused by mutations in the dystrophin gene (Aartsma-Rus et al., 2006; Muntoni, 2003), leading to disruption of the open reading frame (Fig. 2a). Monaco et al. (Monaco et al., 1988) found that frame shift mutations in the DMD gene will lead to a truncated and non-functional dystrophin. Patients with such mutations present less severe Becker muscular dystrophy. This reading frame rule holds true for ~91% of DMD cases (Aartsma-Rus et al., 2006) and has inspired the development of the exon skipping strategy which employs antisense oligonucleotides (AON). These small synthetic RNA molecules are complimentary to exonic or splice site sequences, thereby, upon hybridization, they are able to modulate exon inclusion by the splicing machinery (Manzur et al., 2009; Trollet et al., 2009; van Ommen et al., 2008). Although the functionality of the resulting protein may vary, this treatment could delay or even stop disease progression and improve function in the remaining muscle (Melis et al., 1998; Helderman-van den Enden et al., 2010). The antisense oligonucleotides are chemically modified to resist nucleases and promote RNA binding and are designed to have high sequence specificity. A lot of studies have provided the proof of principle of the therapeutic feasibility of the AON to reframe dystrophin transcripts and restore dystrophin synthesis, both *in vitro* (Aartsma-Rus et al., 2002, 2003, 2004) and *in vivo* using the mdx and DMD mice (Mann et al., 2002; Bremmer-Bout et al., 2004; Heemskerk et al., 2009). In studies in the mdx mouse model, oligonucleotides with chemical properties, similar to the ones of 2'-O-methyl-phosphorothioate (2'OMePS) RNA, were taken up in dystrophin-deficient muscle up to 10 times as much as in healthy muscle tissue, most likely owing to increased permeability of the muscle myofiber membrane. In addition, 4 to 8 weeks' subcutaneous delivery of the oligonucleotides resulted in a steady increase in oligonucleotides levels, exon skipping and dystrophin levels (Heemskerk et al., 2010).

Exon skipping provides a mutation-specific, and so potentially personalized, therapeutic approach for patients with DMD (Fig. 2b). Since mutations cluster around exons 45-55 of DMD, the skipping of one specific exon may be therapeutic for patients with a variety of mutations. The skipping of exon 51 affects the largest subgroup of patients (approximately 13%), including the ones with deletions of exons 45 to 50, 48 to 50, or 52 (Aartsma-Rus et al., 2009). Subsequent clinical trials have shown that two different AON chemistries, either 2'OMePS (van Deutekom et al., 2007) or phosphorodiamidate morpholino oligomer (PMO) (Kinali et al., 2009), targeting DMD exon 51, can restore local dystrophin synthesis in DMD patients with no or minimum side effects. However, some relevant points of pathophysiologic DMD cascade, such as severe muscle wasting, fibrosis and deficient muscle regeneration, may reduce the efficacy of the DMD exon skipping therapy. In addition, as DMD patients suffer from muscle degeneration from their early life, myoblasts undergo extensive division in an attempt to regenerate, eventually leading to exhaustion of the muscle regenerative potential (Yoshida et al., 1998; Hawke et al., 2001; Blau et al., 1985). To overcome these problems, there have been several additional therapies in which myostatin inhibition has received considerable interest (Kemaladewi et al., 2011).

Here we report the three clinical trials based on the exon skipping approach which have been performed to date and we describe the trials that are still ongoing. A study, consisting in the injection of 0.8 mg of PRO051 into the tibialis anterior muscle, was performed by van Deutekom and colleagues (van Deutekom et al., 2007). PRO051 is a 2'OMePS antisense oligoribonucleotide complementary to a 20-nucleotide sequence within exon 51. Four patients with DMD were included in this study and they all had deletions that were correctable by exon-51skipping and had no evidence of dystrophin in the previously made diagnostic muscle biopsy. For every patient mutational status and positive exon-skipping response to PRO051 *in vitro* were confirmed, and T1-weighted MRI was used to determine the condition of the tibialis anterior muscle. The intramuscular injection of PRO051 induced exon-51 skipping, corrected the reading frame, and thus introduced dystrophin in the muscle in all four patients affected by DMD. PRO051 restored dystrophin to levels between 3-12% or 17- 35%, basing on quantification relative to total protein or myofiber content. The poorest results that were obtained in a patient, who had the most advanced disease, led the authors to underline the importance of using patients at a relatively young age, since in them relatively little muscle tissue has been replaced by fibrotic and adipose tissue. Among the adverse effects, mild local pain at the injection site was reported by a patient. Mild-to-moderate pain, after the muscle biopsy, was also reported. Blistering under the bandages used for wound closure was reported by two patients. In the period of time elapsing between injection and biopsy, flu-like symptoms were observed in two patients and a mild diarrhea in a patient.

A dose escalation intramuscular trial was performed in 7 patients (2 patients received 0.09 mg and 5 patients received 0.9 mg of AVI-4658) who received injections in one extensor digitorum brevis (EDB) muscle, while the contralateral EDB muscle was injected with 900 µL normal saline (Kinali et al., 2009). The 7 patients had deletions in the open reading frame of DMD that are responsive to exon 51 and were selected on the basis of the preservation of EDB muscle, as assessed by MRI, and the response of cultured fibroblasts from a skin biopsy to AVI-4658. Muscles were biopsied between 3 and 4 weeks after injection. No adverse events, related to AVI-4658, were reported in this study and they showed an increased dystrophin expression in all treated EDB muscles. Immunostaining of EDB-treated muscle for dystrophin was performed. In the areas of the immunostained sections that were close to the needle track through which AVI-4658 was given, 44-79% of myofibres had increased expression of dystrophin. In randomly chosen sections of treated EDB muscles, the mean intensity of dystrophin staining ranged from 22% to 32% of the mean intensity of dystrophin in healthy control muscles (mean 26.4%), and the mean intensity was 17% (range 11-21%) greater than the intensity in the contralateral saline-treated muscle. In the dystrophin-positive fibres, the intensity of dystrophin staining was up to 42% of that in healthy muscle. Western blot analysis detected increased expression of dystrophin in the AVI-4658-treated muscle of all patients who received the high dose, and the immunoblot detected expression of dystrophin of the expected molecular weight in all patients. This study has led to a dose-ranging systemic study of AVI-4658 in ambulant patients affected by DMD (ClinicalTrials.gov, number NCT00844597).

A phase 1-2a study has been conducted to assess the safety, pharmacokinetics and molecular and clinical effects of systemically administered PRO051 (Goemans et al., 2011). PRO051 was administered subcutaneously for 15 weeks in 12 patients, with each of four possible doses (0.5, 2.0, 4.0, and 6.0 mg per kilogram of body weight) given to 3 patients. Irritation at the administration site and, during the extension phase, mild and variable

proteinuria and increased urinary α (1)-microglobulin levels were reported. The mean terminal half-life of PRO051 in the circulation was 29 days. PRO051, at the dose of 2.0 mg per kilogram or higher, induced specific exon-51 skipping. In 10 patients new dystrophin expression was observed between approximately 60% and 100% of muscle fibers, as observed in post-treatment biopsy. New dystrophin expression increased dose-dependently up to 15.6% of the expression in healthy muscle. After the 12 week extension phase, a modest improvement was observed in the 6 minute walk test (Netherlands National Trial Register number, NTR1241).

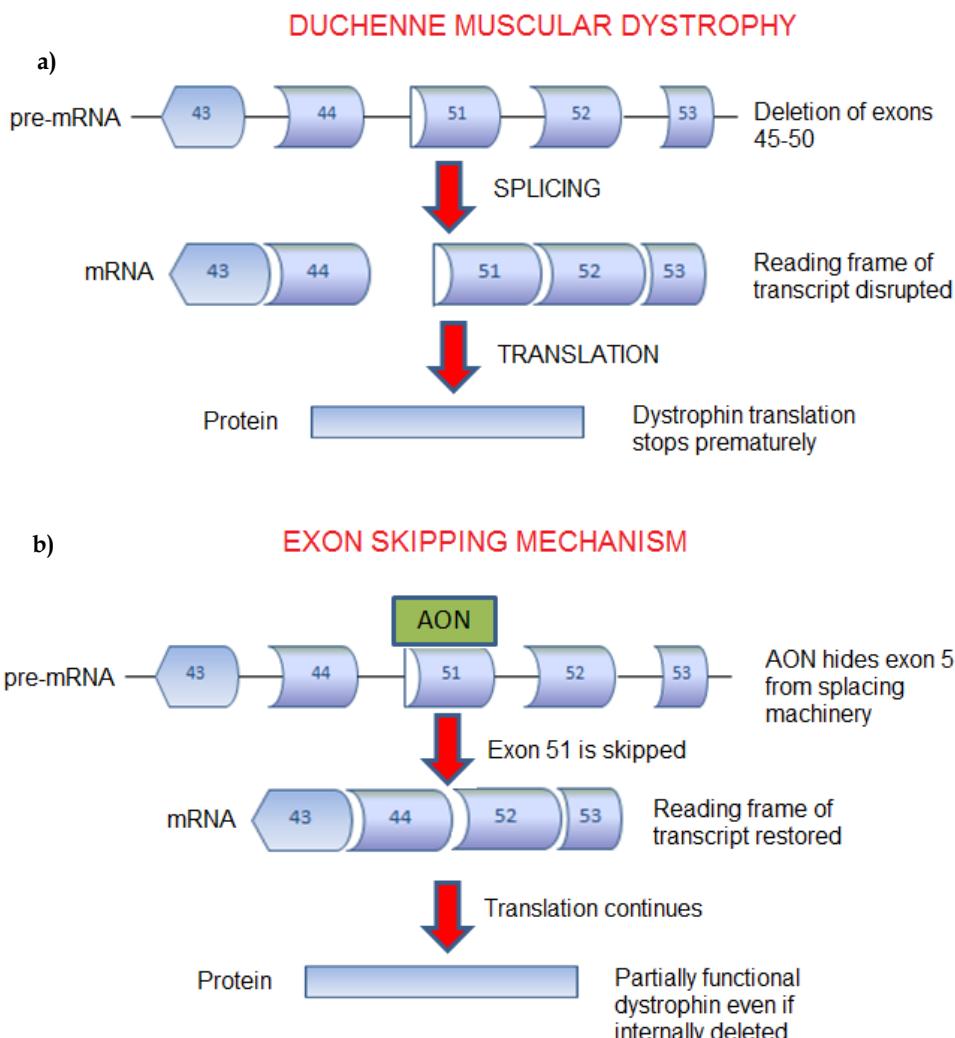


Fig. 2. a) Duchenne muscular dystrophy is caused by mutations in the dystrophin gene, leading to disruption of the open reading frame; b) Exon skipping mechanism in Duchenne muscular dystrophy.

6. Ongoing clinical trials

Several clinical trials, involving DMD patients, and based on the exon skipping approach are ongoing. A phase 1 trial is testing a drug (GSK2402968) that has been designed to skip exon 51 of the dystrophin gene, assessing the safety and tolerability of the drug in boys/adolescents who are unable to walk. The absorption and processing of the drug in their bodies will be also studied. This trial is expected to end in September 2011. A Phase 1/2 trial is investigating whether the experimental drug PRO044 is safe and effective as a therapy for people with DMD with a mutation in a specific region of the dystrophin gene, i.e. exon 44. The expecting end date of the trial is December 2011. Another phase 2 trial is investigating GSK2402968 (two different doses), a drug that has been designed to skip exon 51 of the dystrophin gene. The aim of the study is to determine if an intermittent treatment with GSK2402968 will lead to a better long-term safety profile, while maintaining its effectiveness. This trial is expected to end in September 2012. A phase 3 trial is undergoing testing a drug (GSK2402968) that has been designed to skip exon 51 of the dystrophin gene. It will assess the effect of GSK2402968 on the muscle function of boys with DMD and will monitor the safety of the drug. It is expected to end in December 2012 (The information contained in this paragraph has been collected from the website http://www.musculardystrophy.org/research/clinical_trials/0/duchenne+muscular+dystrophy accessed on 18/05/2011).

7. Cell-based therapy

The cell-based therapy, or cell transplantation, involves different procedures with injected cell pool to correct some functional tissue or organ impairment. Depending on the pathology to be treated, the protocol of cell graft is specific. In genetic disorders, cells are genetically corrected with ex vivo procedure and grafted. In degenerative disorders, cells are amplified and injected. In cancer or infectious pathologies, the cells are selected on the base of immunoreacting or immunomodulating properties, amplified and injected in patients. The cells can derive from other species (xenotransplantation), other subjects (heterologous) or the receiver (autologous). Using cells deriving from other organism, xenotransplantation and heterologous transplantation are associated with the immunosuppressive therapy which reduces host immune-reaction against graft. It is important to decide which cell type to transplant. Based on remaining differentiate ability and plasticity, it is possible to choose the stem cell origin (embryonic, foetal or adult), progenitor cells or terminally committed cells, and collect them from the tissue of interest. Based on remaining differentiated ability and plasticity, it is possible to choose the stem cell origin (embryonic, foetal or adult), progenitor cells or terminally committed cells, from a selected specific tissue and followed by a process and purification before graft. They are frequently amplified and specifically stimulated to increase and improve grafted pool. In autologous transplantation treating genetic disorder, the cells are genetically handled to revert mutation. Depending on localization of the disorder, the cells are locally or systemically injected. The strategies of the cell-based therapy, adopted for DMD, are two: ex vivo genetic correction in autologous cells, followed by graft or heterologous injection of cells. It allows to evaluate rapidly the improvement or disadvantages of the therapy, avoiding severe complications due to impairment in vital muscles. Heterologous transplantation allows to inject low-handled healthy cells with the certainty of avoiding dys

mutations. Unfortunately, the immunosuppressive therapy is necessary to protect graft from host immune system.

8. Cell types

In these last few years, stem cells have received a lot of attention for their potential use in cell-based therapies for various human diseases including DMD (Lodi et al., 2011; Farini et al., 2009). For several years, after they were discovered, the satellite cells were considered as the only cells responsible for the growth and maintenance of the skeletal muscle (Le Grand and Rudnicki, 2007a; Le Grand and Rudnicki, 2007b). With the improvements of cell-isolation technology, a number of markers were described to identify a lot of muscular and nonmuscular subpopulations able to actively participate in myogenesis. Recent works have described the partial identification and characterization of multilineage stem cells derived in culture from numerous adult tissues. In the skeletal muscle itself, rather than satellite cells, alternative adult multi-lineage progenitor cell populations showed to have a myogenic potential: muscle-derived stem cells (MDSCs) (Sarig et al., 2006), muscle side-population (mSP) cells (Wognum et al., 2003) and muscle-derived CD133+ progenitors (Peault et al., 2007). Several works have described how nonmuscular resident stem cells could participate in myogenesis: the bone marrow-derived mesenchymal stem cells (BMMSCs) can differentiate into mesodermal cells, including myoblasts (Pittenger et al., 1999; Prockop, 1997) and adult tissue host cells can also contribute to endodermal and ectodermal cell lineages (Krause et al., 2001; Mezey et al., 2000). A subpopulation of CD133+ cells that play an important role in myogenic development, has been isolated from blood (Torrente et al., 2004). Furthermore, other stem cells have been identified in the dorsal aorta of avian and mammalian species, the so-called mesoangioblasts (Cossu and Bianco, 2003), while the pericytes were found in the basement membrane of the vessels (Dellavalle et al., 2007).

9. Satellite cells

Satellite cells derive from a progenitor population paired box protein 3 and 7 (Pax3 and Pax7, muscle and neural crest development markers) +/+ localized in the central portion of the dermomyotome, the dorso-lateral part of the somite. During the fetal development, the resident progenitor population generates cells in satellite position around myofibers, which are marked by the expression of Pax7, while the limb muscle satellite cells arise from hypaxial cells expressing Pax3. (Le Grand and Rudnicki, 2007b). The satellite cells are located beneath the basal lamina of mature skeletal muscle fibres, and they are ideally positioned to repair degenerating muscle fibres. These quiescent cells are activated to proliferate upon muscle injury or when heavily used during activities such as weight lifting or running. This proliferation step is necessary to generate sufficient numbers of myoblasts for muscle differentiation and myotube formation. In humans, these mononuclear cells are most plentiful at birth (estimated at 32% of sublaminar nuclei). Their frequency declines postnatally, stabilizing between 1% and 5% of skeletal muscle nuclei in the adult muscle. In humans, the proportion of satellite cells in skeletal muscles also decreases with age, and it could explain the decreased efficiency of muscle regeneration in older subjects. Satellite cells from aged muscle also display reduced proliferative and fusion capacity, as well as a tendency to store fat, thus deteriorating their regeneration potential. Satellite cells present an

extended proliferative potential and can repopulate extensively the host's muscle with an efficiency unknown in any other experimental situation (Cooper et al., 2006).

10. Multipotent muscle-derived stem cells

Recent studies have demonstrated the existence of a population of multipotent muscle-derived stem cells (MMDSCs), distinct from satellite cells, with high myogenic potential in vitro, even after being appropriately stimulated to differentiate into other lineages, such as haematopoietic. MMDSCs reside in skeletal muscle sharing the ability to self-renew and differentiate into other mesodermal cell types (Farini et al., 2009). MDSCs were isolated on the base of their adhesion ability. The cells, obtained by enzymatic digestion of muscle tissue, are seeded on a culture dish and, after 1 hour, the medium and non-adherent cells are transferred to another dish (preplating). Then, analogous preplates are repeated at 24 hour intervals until preplate 6 (pp6) is completed. The cells, which rapidly attach to the surface, are mainly fibroblasts (pp1), cells which adhere within 24-48 hours. They are predominantly satellite cells (pp2 – pp4) and the population, which settle at the most slowest speed on a flask, consists of multipotential stem cells (pp-6). The phenotype of fraction pp6 is described as stem cell antigen-1 (sca-1), CD34+ (marker of hematopoietic and satellite cells), CD45-, c-kit- (markers of hematopoietic cells) with the expression of desmin (marker of myogenic cells) on a different level. MDSCs, cultured in vitro, differentiate spontaneously into myotubes but, when appropriately stimulated, these cells can also give rise either to osteoblasts, chondroblasts, hematopoietic cells or endothelial cells. MDSCs firmly adhere to endothelium in mdx muscles microcirculation and then participate in muscle regeneration, following an intramuscular injection. Interestingly, they have also been found in muscles, after intravenous administration, and their number was higher in muscles, previously injured, than in the control ones. The expression of desmin gradually decreases with subsequent preplates and in pp6 population only about 10-20% of cells are positive for this protein. However, pp6 cells, cultured in standard conditions, spontaneously enter the myogenic pathway that is associated with an increased desmin expression. It has also been demonstrated that the majority of MDSCs is positive as for desmin. The studies, regarding cell viability after transplantation, have shown that more MDSCs survive following intramuscular administration, if compared to more differentiated cells (myoblasts). After the injection of the same number of either MDSCs or early preplate (EP) cells, the contribution to muscle regeneration, 30 and 90 days later, has been even 10 times higher in MDSC group. Marked differences have probably been associated with the distinct immunogenicity between MDSCs and EP cells. The evaluation of major histocompatibility complex (MHC-1) expression in cell membranes of both cell types has revealed that MHC-1 is present in 63% of EP cells, whereas only in 0.5% of MDSCs. However, MDSCs are a much less numerous cell population if compared to "typical" satellite cells which dominate in EP group. Only one clone of MDSCs can be obtained from 10^5 of cells originally isolated from muscle tissue (Lee-Pullen and Grounds, 2005). MMDSCs could also be distinguished by flow cytometry. After staining with Hoechst 33324, it was possible to characterize a cell population, called skeletal muscle side population (SMSP) or simply side population (SP), which is able to extrude dye via ATP-binding cassette G2 (ABCG2) multi-drug resistant pump. SP expresses several surface markers associated with haematopoietic stem cells (HSCs) including CD45, c-kit, Sca-1 and CD34, but no satellite cell markers such as M-cadherin, Pax7 or desmin. SP cells can give rise to all hematopoietic lines, both in vitro and in vivo. The question of myogenic

potential is more complex. The SP cells, harvested in vitro, do not differentiate spontaneously into myocytes. However, cell-mediated inductive interactions trigger myogenic potential of SP cells. These cells contain two distinct fractions with myogenic potential, such as CD45+ and CD45-, both exhibiting the potential to constitute myogenic cells after a co-culture with primary myoblasts. In particular, CD45+ SP are able to integrate into regenerating muscle fibres after an intramuscular injection, while the sub-fraction CD45- SP has the potential to give rise to adipocytes and osteocyte. Regardless of hematopoietic and myogenic potential, SP also displays endothelial trait (CD31+). Moreover, it has been shown that SP expresses angiopoietin 2 and the Tie2 receptor, which is bound and activated by angiopoietins. It means, that most of the SP cells share partially signalling pathways with endothelial/hematopoietic precursor cell populations. SP cells demonstrate that lack of Pax7 gene in experimental animals does not influence the number of muscle SP cells. Furthermore, Pax7-/- SP cells can differentiate into myotubes when co-cultured with myoblasts. It indicates that Pax7 gene is not required for myogenic specification of SP cells. Finally, forced expression of MyoD induces myogenic differentiation of Pax7-/- SP cells, but not pax7-/- myoblasts. All these data suggest that SMSP and satellite cells are distinct populations and probably have different origin (Burdzinska et al., 2008).

11. Blood- and muscle-derived CD133+ progenitors

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. Circulating human CD133+ cells demonstrate stemness properties and the ability to restore dystrophin expression and eventually regenerate the satellite cell pool in dystrophic scid/mdx mouse after intra-muscular and intra-arterial delivery. Skeletal muscle CD133+ stem cell have the potential to differentiate towards both muscle and endothelium lineages. Dystrophic human CD133+ are able to express an exon-skipped version of human dystrophin, after transduction with a lentivirus, carrying a construct designed to skip exon 51; therefore their ability to participate in muscle regeneration has been examined after transplantation into scid/mdx mice. The comparison of two distinct CD133+ cell populations, one from blood and one from skeletal muscle, show that the muscle-derived CD133+ stem cells have the potential to differentiate towards both muscle and endothelium lineages. Skipped blood and muscle-derived Δ49–50 stem cells, fused in vivo with regenerative fibres, express a functional human dystrophin and restructure the dystrophin-associated protein complex, as shown by plasmalemma re-expression of a and b-sarcoglycan proteins. Moreover, being sometimes located beneath the basal lamina, and distributed along freshly isolated fibres, it is interesting to assess whether muscle-derived CD133+ stem cells are able to differentiate into satellite cells. Genetically engineered DMD muscle-derived CD133+ cells show a better muscle regeneration in terms of spreading and number of positive fibres in comparison with the results obtained from blood-derived stem cells. DMD muscle-derived CD133+ cells are more efficient than their blood counterpart in the improvement of morphology and restoration of the normal skeletal muscle function in dystrophic murine muscles. Human CD133+ cells, isolated from muscle or blood, are able to promote muscular- and endothelial-differentiation after intra-arterial and intra-muscular delivery. These cells can be injected safely in DMD patients, not only without side effects, but also promoting an increase in the number of capillaries per muscle fibres. Moreover, DMD CD133+ cells can be genetically modified to re-express a functional dystrophy. Unfortunately, several things need to be

ameliorated, such as the potential to enhance proliferation of blood-derived CD133+ cells in culture and storage for repeated treatments, the relative efficiency of blood-derived cells, compared with muscle-derived cells to contribute to muscle nuclei, the strategy to deliver myogenic cells chronically to the various sites of sporadic regeneration that occur in muscular dystrophies (Farini et al., 2009; Peault et al., 2007).

12. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are conventionally defined as adherent, non-hematopoietic cells expressing markers such as CD90, CD105, and CD73, and being negative for CD14, CD34, and CD45. While originally identified in the bone marrow, MSCs have been extracted from numerous tissues including adipose tissue, heart, Wharton's jelly, dental pulp, peripheral blood, cord blood menstrual blood, and, more recently, Fallopian tube. One of the major properties of MSCs is the ability to differentiate into various tissues. The traditional differentiation properties of MSCs are their ability to become adipocytes, chondrocytes, and osteocytes *in vitro*, after treatment with induction agents. Non-orthodox differentiation into other tissues, for example, cells resembling neurons, muscles, hepatocytes and pancreatic islets, has also been reported. There is some evidence that MSCs may differentiate selectively into tissues that have been injured. The ease of obtaining bone marrow sample and myogenic potential of MSCs makes this population an attractive candidate for cellular transplantation in cases of diseases associated with muscle dysfunction. However, there are still a lot of controversies around the level of myogenic potential of mesenchymal cells. Numerous studies were focused on the methodology of induction of MSC differentiation into muscle cells. Contrasting results were obtained treating MSCs with DNA methyltransferase inhibitor, such as 5-azacitidine, or galectin-1 as the factor initializing myogenesis of MSCs (Chan et al., 2006; Liu et al., 2003). Regarding BMMSC myogenic potential, interesting results were achieved culturing the cells by a mixture of cytokines and growth factors (fibroblast growth factor beta (β FGF), forskolin, platelet derived growth factor (PDGF), neuregulin and subsequently transfected with gene encoding Notch 1 intracellular domain (NICD). Following this procedure, cells differentiated into muscle cells with the efficacy of 89% (Dezawa et al., 2005). An alternative approach to induce myogenesis in MSCs, is the exposure of these cells to myogenic environment. MSCs, co-cultured with cardiomyocytes or satellite cells, differentiated into either cardiac cells or myotubes respectively (Fukuhara et al., 2003; Lee et al., 2005). However, the differentiation rate in these conditions was highly limited. The fate of MSCs, injected into either skeletal or cardiac muscle, was also analyzed (Shi et al., 2004). It has been demonstrated that undifferentiated MSCs can undergo myogenesis after an intramuscular administration but, similarly to the *in vitro* studies, the proportion of differentiated cells was barely detectable, only 0.44% of transplanted MSCs fused in myotubes. Gene-corrected DMD MSCs restored dystrophin expression in co-cultured dystrophic myoblasts through spontaneous cell fusion (Goncalves et al., 2006a). Furthermore, a study where dystrophic MSCs transfected by recombinant adenovirus, which contains human microdystrophin cDNA, were injected into *mdx* mouse, showed that expression of dystrophin was detected in dystrophic tissue (Xiong et al., 2007). A study has also compared MSCs transplantation with and without prior differentiation. Cells were injected around the myocardial infarcted area of a rabbit model. The improvement in left ventricular function, vascular density and reduction of infarcted area did not differ significantly between the two groups. The perspective to transplant

undifferentiated mesenchymal stem cells seems to be promising because it does not require time-consuming and expensive extracorporeal manipulations in cells (Ichim et al., 2010).

13. Mesoangioblasts

Mesoangioblasts are multipotent progenitors of mesodermal tissues, physically associated with the embryonic dorsal aorta in avian and mammalian species. Mesoangioblasts are able to differentiate into various mesodermal phenotypes. Mesoangioblast-like cells have been isolated from vessels of post-natal tissues. Post-natal cells generally express pericytes rather than endothelial cell markers, but they are otherwise similar to their embryonic counterparts in terms of proliferation and differentiation potency. When wild-type or dystrophic, genetically corrected mesoangioblasts are delivered intra-arterially to dystrophic muscle of α -sarcoglycan- null mice (a model for limb girdle muscular dystrophy), they induce a dramatic functional amelioration of the dystrophic phenotype. This is due to the widespread distribution of the donor's cells through the capillary network and to an intrinsic defect of proliferation in the resident satellite cells, a situation that creates a selective advantage for the injected donor cells. To proceed with clinical experimentation, it has been considered to be crucial that the delivery and muscle homing of mesoangioblasts may be optimized to characterize human cells in depth and the protocol needs to be tested in a large animal model. Recently, it has been reported that the enhancing delivery of mesoangioblasts leads to the complete reconstitution of downstream skeletal muscles in α -sarcoglycan-null dystrophic mice. Mesoangioblasts, exposed *in vitro* to either stromal cell derived factor-1 or TNF- α , have showed enhanced transmigration *in vitro* and migration into dystrophic muscle *in vivo*. Transient expression of α -4 integrins or l-selectin have also produced a several-fold increase in migration, both *in vitro* and *in vivo*. Mesoangioblasts, transduced with a lentiviral vector expressing human microdystrophin and injected scid/mdl mice and immunosuppressed dystrophic golden retriever muscular dystrophy (GRMD) dogs, have showed a modified mesoangioblasts-induced dystrophin positivity in myofibres. In particular, the results of these injections have been promising in the dystrophic dogs with improvements in their muscle function and mobility together with an increased dystrophin expression. In order to ameliorate the efficiency of the muscle repair by mesoangioblasts, cell migration to skeletal muscle has been improved and unspecific trapping in the capillary filters of the body, such as liver and lung, has been reduced (Farini et al., 2009).

14. Bone marrow stem cells

In the last decade, it has been discovered the contribution of various nonmyogenic cells in the regeneration of skeletal muscle, such as bone marrow-derived cells (BMDCs) and the circulating haematopoietic cells. With the advent of more sensitive markers, it has been demonstrated that BMDCs can enter the sites of muscle regeneration and also contribute to the formation of new muscle fibres. Furthermore, similar cells, resident in skeletal muscle, appeared to reconstitute the bone marrow and, via this route, enter again and contribute to the regeneration of skeletal muscle. In this sense, it has been demonstrated that the intravenous injection of either normal HSCs or a novel population of MDSCs into irradiated mdx mice resulted in the reconstitution of the haematopoietic compartment of the transplanted recipients, the incorporation of donor-derived nuclei into muscle, and the partial restoration of dystrophin expression in the affected muscle. Similarly, after the

transplantation into immunodeficient mice, BMDCs migrated into areas of induced muscle degeneration, underwent myogenic differentiation, and participated in the regeneration of the damaged fibres. Following the transplantation in irradiated mice ablated of endogenous satellite cells, the transplanted BMDCs were able to occupy the niche of those satellite cells. Furthermore, BMDC satellite cells participated in the regeneration of multinucleated muscle fibres at high frequency, becoming heritably myogenic. As these results were obtained using the whole bone marrow as transplant source, and since it is known to contain haematopoietic and non-haematopoietic progenitors, it is possible to speculate that the bone marrow could contain such progenitors for muscle and blood. On the other hand, a common progenitor with haematopoietic potential could generate myogenic cells due to either physiological stimuli and fusion with a myogenic cell (Farini et al., 2009).

15. Pericytes

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 the adult bone marrow under ischemic conditions, and utilized for their contractile capabilities and their multiple cytoplasmic processes. It has been demonstrated that pericytes have a high capacity of myogenic differentiation because they give rise to a high number of muscular fibres, when injected into scid/mdx mice. It has been proposed that the pericyte could be released from its position on a vascular tube in the case of a focal injury, functioning as an immunomodulatory and trophic mesenchymal stem cell. The activity of the pericyte ensures that the field of damage remains limited and that tissue-intrinsic progenitors replace the expired cells. As provided by this evidence, these stem cells could represent a good candidate for the muscle therapy because they could be isolated from a muscle biopsy and therefore 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. Nevertheless, it would be important to determine whether transplanted pericytes can fully reconstitute the satellite cell niche as a real functional stem cell. More information are needed about the role of pericytes, in both normal and dystrophic skeletal muscle, in order to avoid that the injection of these cells into human dystrophic muscle environment could elicit pericyte-derived tumours.

16. Adipocytes

Adipocytes share the same mesodermal origin with skeletal muscle. An inverse relationship, between skeletal muscle mass and adipose tissue mass, is apparent in murine models of skeletal muscle dystrophic pathology such as mdx where the relative level of fat tissue, within the diseased muscle, has increased. Moreover, in the myostatin-/- mouse, where the skeletal muscle mass has hugely increased, the fat tissue mass is reduced substantially. Moreover, cell culture studies have demonstrated that myogenic cell lines, when made to overexpress adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer-binding protein alpha (C/EBP α) lose their myogenic marker expression and differentiate into adipocytes, suggesting that the adult myoblasts are capable of being reprogrammed to become adipocytes. Furthermore, the satellite cell population has been shown to be capable of conversion into the adipocyte

lineage, given the correct cues. A more recent work has provided good evidence that the adipocyte lineage can also undergo conversion into myoblasts. An adipocyte-specific stem cell population that expressed high levels of CD13, CD44, CD73 and CD90 and was negative for CD34, CD45, CD56 and CD184, suggesting mesenchymal stem cell-like characteristics, was recently isolated and has shown to display myogenic markers and fuse with maturing myofibres when co-cultured with myoblast cell lines. Furthermore, it has been demonstrated that cells, isolated from the stromal vascular fraction of adipose tissue, which have been shown to differentiate *in vitro* into adipogenic, chondrogenic, osteogenic and myogenic cells, can spontaneously form myotubes when cultured under standard conditions *in vitro*. Furthermore, these cells are able to fuse into myotubes following *in vitro* expansion and following injection into ischaemic murine hind limbs. They also form new myofibres and are capable of restoring dystrophin expression in mdx mice, thus displaying a therapeutic potential. More recently, a more specific CD45- side population of adipocyte progenitor cells, purified from the stromal vascular fraction, has been shown to form myofibres *in vivo*. An array analysis of murine brown fat precursor cell populations has recently shown that certain myogenic transcription factors, including myogenin, Myf5 and MyoD, are expressed at levels comparable with C2C12 cells within these progenitors. These data are in keeping with the previous lineage-tracing studies that showed a dermomyotomal origin for brown, but not white, fat precursor cells. Moreover, brown fat lineage cells also express the known myogenic microRNAs miR-1 miR-133a and miR-206, suggesting that brown adipocytes share a common ancestor with myogenic cells. Subsequently, it has been demonstrated that the transcription factor PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16), is sufficient and necessary to drive the conversion of white adipocytes into brown adipocytes, through an up-regulation of uncoupling protein and PPAR γ coactivator-1alpha (PGC1- α) expression. It shows to be a key regulator in the formation of the brown fat lineage. PRDM16 drives the brown fat lineage by forming a transcriptional complex with C-terminal binding protein 1 or 2, whereby it acts to repress white fat-specific genes or complexing with PGC1- α , and PPAR γ Coactivator 1-beta (PGC1- β) according to which it enhances expression of brown fat genes. Recently, it has been elegantly shown that brown fat cells arise from a Myf5+ common precursor that was previously only thought to form skeletal muscle cells. Furthermore, PRDM16 overexpression causes brown fat cells to undergo a lineage switch, forming skeletal myoblasts through the activation of PPAR- γ , whereas PRDM16- / brown fat has elevated myogenic gene transcription and reduced uncoupling ability. Subsequently, it has been shown that human skeletal muscle contains a population of brown fat precursor cells that up-regulates uncoupling protein 1 following PPAR- γ agonist treatment. Finally, human adipose tissue-derived mesenchymal stem cells have been shown to differentiate into myofibres spontaneously as well as induce dystrophin expression following co-culture with human DMD myoblasts *in vitro* through a cell fusion. These data show that adipogenic stem cells may be used for therapeutic applications (Otto et al., 2009).

17. Clinical trials

Myoblast transplantation is a possible treatment for DMD. Promising results *in vivo* nude/mdx mouse transplantation was obtained and in the 90's a series of clinical trials on DMD patients was conducted. Huard et al. (Huard, et al. 1991; Huard, et al. 1992) transplanted myoblasts from an immunocompatible donor into the limb muscles of 4 DMD

patients in the advanced stages of the disease. A different degree of dystrophin was detected by immunostaining in the patients, but this change slowly decayed over time and it was not associated to a strength improvement. Although no immunosuppressive treatment was used, no patient showed any clinical sign of rejection. The effects of myoblast transplantations, without an immunosuppressive treatment on muscle strength and the formation of dystrophin-positive fibers, were studied in five young boys with DMD, using a triple blind design. No increase in the static contraction was detected. The expression of dystrophin in myoblast-injected fibers was generally low and it decreased to control level in 6 months. These results strongly suggest that myoblast transplantations, as well as gene therapy for DMD, cannot be done without immunosuppression (Tremblay et al., 1993). Karpati et al. (Karpati et al., 1993) used cyclophosphamide as an immunosuppressive agent to improve the myoblast transfer; however, subsequent experiments demonstrated that this antitumour drug killed the transplanted myoblasts, as well as any other rapidly proliferating cells. Normal dystrophin was detected, by reverse-transcriptase polymerase chain reaction, in DMD patients after myoblast transplantation, but it was not associated to an increase in the percentage of dystrophin-positive fibers (Gussoni et al., 1992). Immunosuppression of DMD boys by cyclosporine, during myoblast transplantation, improved force generation, but it was not effective in replacing clinically significant amounts of dystrophin in DMD muscle (Miller et al., 1997). Mendell et al. (Mendell et al., 1995) injected myoblast, once a month for six months, in 12 DMD patients, but this treatment failed to improve strength. Law et al. (Law et al., 1992) demonstrated the feasibility and safety of myoblast transplantation, but with a poor clinical improvement. At the end of the 90's, a careful overview of the previous initial trials brought several research teams to identify three problems responsible for the limited results observed: (1) 3 days after the graft, at least 75% of the transplanted myoblasts died (Fan et al., 1996; Guerette et al., 1997; Huard et al., 1994); (2) myoblasts were not able to migrate more than 200 μm away from the intramuscular injection trajectory (Skuk et al., 1999); (3) if immunosuppression was not adequate, the myoblasts were rapidly rejected in less than 2 weeks (Guerette et al., 1994) or were induced to activate apoptosis, such as cyclophosphamide usage (Hardiman et al., 1993; Hong et al., 2002). There are now some solutions to overcome these problems. The rapid death of a large percentage of myoblasts can be compensated by the transplantation of a high number of cells. Indeed the transplantation of 30 million cells per mm³ has given very good results in monkeys (Skuk et al., 1999). In monkeys, the low migration distance of myoblasts was avoided by a high number of adjacent intramuscular injections, i.e. 100 injections per cm² of muscle surface. High density of intramuscular injections of myoblast in 11 DMD patients were also well tolerated. One patient received a total of 4,000 intramuscular injections without any complication. No infection or other complication, related to the procedure, was registered (Skuk et al., 2007). An interesting improvement in myoblast migration injected in muscle was obtained by the modulation of MyoD expression (El Fahime et al., 2000; Smythe and Grounds, 2001). Similarly, it has been observed the restoration of 26–30% dystrophin expression in muscle fibers in the environment of the irradiated muscle, suggesting an improvement in myoblast migration induced by factors released with the irradiated muscle (Cousins et al., 2004; Skuk et al., 2006; Skuk et al., 2007; Skuk et al., 2004). The immunosuppression problem was tackled introducing new drugs. FK506 (Tacrolimus or Prograf®; Astellas Pharma, Deerfield, IL, USA) allowed to obtain very good transplantation results, not only in mice, but also in monkeys (Kinoshita et al., 1996; Kinoshita et al., 1994). Unfortunately, FK506 may induce adverse effects in patients

(nephrotoxicity, diabetes, increased risk of cancer) if used on an ongoing, long-term basis (Palmieri et al., 2010). Many therapeutic protocols were developed to induce specific immunological tolerance towards the donor's myoblasts and through the creation of mixed chimerism and central tolerance (Camirand et al., 2004; Stephan et al., 2006). Another support to immunosuppression therapy was the transplantation of genetically modified autologous myoblasts (Floyd et al., 1998; Goncalves et al., 2006b; Quenneville et al., 2007), or avoidance of gradual senescence of differentiated cells, autologous pluripotent stem cells (Di Rocco et al., 2006). Other researchers refocus their efforts to optimise the therapy searching cell populations thought to be more primitive and less immunologic than myoblasts. They include MDSCs (Sarig et al., 2006), mSP cells (Wognum et al., 2003) and muscle-derived CD133+ progenitors (Farini et al., 2009). In a double-blind phase I clinical trial, Torrente et al. (Torrente et al., 2007) transplanted autologous CD133+ cells, extracted from muscle biopsies by intramuscular injection, into eight boys with DMD and sampled after 7 months. The cells were not genetically corrected, their fate was not monitored, and the boys were not immunosuppressed, because the experiment was designed only to test the safety of the implanted cells (grown in culture for only 48 hours). No adverse effects were observed. Afterwards, thanks to these observations, the cell therapy was combined with a genetic approach: ex vivo introduction of corrective genes into dystrophic CD133+ myogenic cells and their subsequent autologous transplantation. The use of exon-skipping for the expression of human dystrophin, within the DMD CD133+ cells, allows the use of the patient's own stem cells, thus minimizing the risk of immunological graft rejection (Riviere et al., 2006). The exon-skipping therapeutic approach is applicable to gene defects up to 70% of DMD patients, and avoids the problems associated with the delivery of the prohibitively large full length dystrophin gene or a (less functional) truncated mini-gene. Blood- and muscle-derived DMD CD133+ cells were isolated and characterized for their ability to express an exon-skipped version of human dystrophin, after infection with a lentivirus carrying a construct designed to skip exon 5 (Denti et al., 2006; Goyenvalle et al., 2004). The skipped blood and muscle-derived Δ49–50 stem cells were able to fuse in vivo with regenerative fibres and expressed, not only a functional human dystrophin, but also the dystrophin-associated proteins a and b-sarcoglycans. However, intramuscular transplantations lead only to local and focused regeneration, whereas DMD pathology affects the whole body musculature and its effective treatment requires some methods to distribute the injected cells to the dispersed sites. In future clinical trials, we speculate that these stem cells, purified from DMD patients, could be ex vivo engineered and reinjected in the initial donor intra-arterially. The intra-arterial injections of the patients' own infected stem cells allow the distribution of the cells to the whole body musculature so that it could be possible to take care of severely affected patients that have a reduced body mass. One of the most important problem to solve, for a future clinical application, is the amelioration in safety procedures of the gene modifications. Ichim et al. (Ichim et al., 2010) reported the case study of a 22 year-old male diagnosed with DMD, treated with a combination of endometrial regenerative cells (ERC) and CD34+ umbilical cord blood. Three months later, the patient received another course of therapy including placental matrix derived mesenchymal stem cells. The improvement in muscular strength, clinical respiratory function and general level of activity are maintained to date. No adverse events have been associated with the stem cell infusion. The innovation introduced by this trial was the usage of "adjuvant" cellular population which provides a more suitable environment for muscle regeneration. The local intramuscular MSCs are able to add chemotactic/ trophic support for the intravenously administered CD34. It has been

reported that mesoangioblasts, which reside within the CD34 population, as well as cord blood CD34 cells, have had positive activity on DMD in animal models, although they appear to be short-lived (Jazedje et al., 2009; Nunes et al., 2007; Otto et al., 2009). Mesoangioblasts have been the main contributors to de novo myogenesis and were selectively attracted into the dystrophic tissue. It is known that CD34 cells express very late antigen-4 (VLA-4), which is the ligand for Vascular Cell Adhesion Molecule 1 (VCAM-1), whose expression is elevated in dystrophic muscles (Gavina et al., 2006). Furthermore, CD34 chemokines such as Macrophage inflammatory protein-1 alpha (MIP-1 α) and Regulated upon activation normal T-cell expressed and secreted (RANTES) expression have been found in dystrophic muscle (Demoule et al., 2005).

18. Conclusions

According to this review, the attempts to treat DMD with different genetic and cellular approaches open new perspectives in the muscular function restoration, although it is very difficult to predict, at the moment, what strategy would be more successful. From a lifelong perspective, it is reasonable to conceive an integrated schedule of treatment protocols, subdivided by decades of age. On this basis, cell transplantation is supposed to be the final answer to replace the irreversibly impaired muscle mass with healthy myocytes. It is therefore important to choose the best age for the transplant and the best timing schedule is probably before the crash of the muscular framework that will be replaced with fibrous and adipose tissue. Alternatively, in the very early period of life, when the disease is not phenotypically expressed yet, and it is just a genetic biochemical trait, the timing could be perfect for either exon skipping or cell transplant approaches that could be more easily integrated in the muscular structure. Furthermore, in the homologous cell transplantation, a longstanding immunosuppressive phase, which is strongly adversed by the ethical committees, is at the moment mandatory in order to achieve an adequate survival of nonidentical transplanted cells. These cells should be reasonably replaced with a series of transplant sessions in the follow up, as long as their vital cycle will decline to apoptosis. Probably, in the next future, even histocompatible cadaver sources might be helpful in the long run.

As to the cell administration route, the transplant procedure, addressed by Cossu and coworkers (Sampaoli et al., 2003; Díaz-Manera et al., 2010), through intra-arterial regional perfusion, has to be validated and compared with the more cumbersome repeated intramuscular injections for which automatically injecting devices, a proper anesthesia and, probably, laparoscopic-thoracoscopic schedule will be required, especially for the deep muscles, like diaphragm and heart. In the procedure described by Cossu (Sampaoli et al., 2006), according to the results obtained in dog models of DMD, the mesangioblasts can easily trespass the endothelial barrier and reach the muscular areas to be restored by new healthy myocytes. On the other hand, in the direct intramuscular injections, the operator chooses the topography of injections, and, on the basis of mathematical and geometrical models, and with the hopeful aid of robotization, a systematic and well arranged replacement of several millions of myocytes can be achieved, during each session, on a very accurately, individually tailored protocol. The exon-skipping approach is very appealing due to the progress observed in the clinical trials, but it is very hard to conceive that this genetic correction of the dystrophin gene will be enough effective to support the muscular

strength in the developing child, guaranteeing long lasting surrogate effect if introduced in a lifelong administration schedule. In this scenario, the oral route is certainly preferred to the subcutaneous one, and toxicological studies in mice let us suppose that the compounds are safe, but clinical trials in humans require further commitments, especially regarding the clinical monitoring of the follow up to determine possible side effects.

In conclusion, we think that, at the moment, due to brand new strong technological weapons, we could achieve a normal life span and quality of life for DMD kids and their families. At the same time, we need the collaboration of the centers working on DMD to design new clinical trials in order to reach the best effective therapeutic protocol as soon as possible. In the "Global Village", based on the World Wide Web, it is possible to plan panels of evidence-based medicine clinical studies by a single international World Wide Web based committee, eventually organized by the World Health Organization. This committee will be a powerful instrument to meet the urgent demand of the generation of kids with DMD who claim to achieve the goal of a fully autonomous life.

19. Statement of authorship

The authors hereby certify that all work contained in this review is original work of Tommaso Iannitti, Daniele Lodi, Valeriana Sblendorio, Valentina Rottigni and Beniamino Palmieri. All the information, taken from other articles, including tables and pictures, have been referenced in the "Bibliography" section. The authors claim full responsibility for the contents of the article.

20. Conflict of interest statement

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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22. Abbreviations

- 2'OMePS: 2'-O-methyl-phosphorothioate
- ABCG2: ATP Binding Cassette G2
- AON: Antisense Oligonucleotide
- BMDC: Bone Marrow Derived Cell
- BMMSC: Bone Marrow Mesenchymal Stem Cell
- β FGF: Fibroblast Growth Factor beta
- C/EBPa: CCAAT/Enhancer Binding Protein alpha
- CNS: Central Nervous System
- CsA: Ciclosporin A
- DGC: Dystrophin Glycoprotein Complex
- DMD: Duchenne Muscular Dystrophy
- Dp: Dystrophin proteins
- Dys: Dystrophin

ECM: Extracellular matrix
EDB: Extensor Digitorum Brevis
EP: Early Preplate
ERC: Endometrial Regenerative Cell
GRMD: Golden Retriever Muscular Dystrophy
HSC: Haematopoietic Stem Cell
IQ: Intelligence Quotient
MDSC: Muscle Derived Stem Cell
MHC: Major Histocompatibility Complex
MIP-1 α : Macrophage Inflammatory Protein-1 alpha
MMDSC: Multipotent Muscle Derived Stem Cell
MRI: Magnetic Resonance Imaging
MSC: Mesenchymal Stem Cell
mSP: muscle Side Population
MVC: Maximum Voluntary Contraction
N: Naples
NICD: Notch1 Intracellular Domain
NTR: Netherlands National Trial Register
PDGF: Plateled Derived Growth Factor
PGC1- α : PPAR γ Coactivator 1-alpha
PGC1- β : PPAR γ Coactivator 1-beta
PMO: Phosphorodiamidate Morpholino Oligomer
PPAR γ : Peroxisome Proliferator Activated Receptor gamma
pp6: preplate 6
RANTES: Regulated upon Activation, Normal T-cell Expressed and Secreted
SMSP: skeletal muscle side population
SP: Side Population
T: Toronto
TGF- β : Transforming Growth Factor beta
TNF- α : Tumor Necrosis Factor-alpha
VCAM-1: Vascular Cell Adhesion Molecule 1
VLA-4: Very Late Antigen-4

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