Refinement of antisense oligonucleotide mediated exon skipping as therapy for Duchenne muscular dystrophy

Heemskerk, J.A.

Chapter 1

General introduction

1.1 Duchenne muscular dystrophy

Aetiology and pathology

In 1852 Edward Meryon was the first to describe the severe muscle wasting disease we now know by the name of Duchenne muscular dystrophy (DMD) (Meryon, 1852). It was named after the man who made a detailed description 16 years later, Guillaume Duchenne (Duchenne, 1868). Early symptoms are muscle weakness and hypertrophy of the calf muscles, apparent around the age of four. Within a few years, patients have difficulty walking and need Gowers' maneuver to stand up (figure 1, Gowers), early in their second decade they lose the ability to walk or stand altogether. Without treatment, patients die of respiratory failure around the age of twenty. However, with current treatment, especially assisted ventilation, most patients in the western world make it into their third decade. The heart muscle is also affected in DMD and nowadays 10-30% of patients die of heart failure (Cox and Kunkel, 1997).

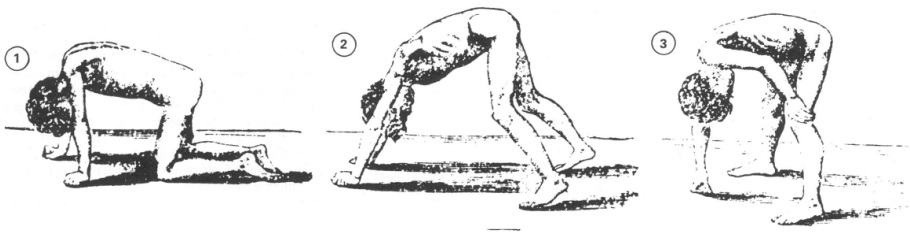


Figure 1: Gowers' manoeuvre. Standing up by 1. getting on hands and knees, followed by 2. stretching the legs and 3. 'walking' the hands up the upper legs, is a typical sign of Duchenne muscular dystrophy. (Drawings from Gowers, Pseudo-hypertrophic muscular paralysis: a clinical lecture, 1879, J A Churchill, London)

One in every 3500 newborn boys is affected by the disease, which proved to be monogenic and is caused by mutations in the X-chromosomal 2.2 Mb *DMD* gene (Koenig et al., 1987), the biggest human gene known to date. The protein it encodes is called dystrophin, is located at the cell membrane, better known as sarcolemma (Wakayama et al., 1993), and can be divided in four domains: the N-terminal domain, the central rod domain, the cysteine-rich domain and the C-terminal domain (figure 2) (Hoffman et al., 1987; Blake et al., 2002). The N-terminal domain and elements of the central rod domain interact with cytoskeletal actin; the N-terminal part binds actin tightly, while repeats 11-17 in the central rod domain have a looser electrostatic interaction (Rybakova et al., 2006). Through spectrin-like repeats 1-3 in the central rod domain, dystrophin also interacts with phospholipids in the sarcolemma (Legardinier et al., 2008). Further, the spectrin-like repeats in the central rod domain give the

protein elastic properties (Mirza et al., 2010). The cysteine rich and C-terminal domains interact with a series of proteins at the sarcolemma to form the dystrophin-glycoprotein complex (DGC) (Judge et al., 2006). Through β- and α-dystroglycan in the DGC, dystrophin is connected to the sarcolemma and the extracellular matrix (ECM). This connection, plus the interaction with actin and the elastic properties, makes dystrophin into a shock absorber (Ervasti, 2007) and stabilize muscle fibers during contraction. If one of the two actin binding parts is deleted this effect is partly lost, but compared to DMD, the phenotype is mild (Corrado et al., 1996, also see Becker muscular dystrophy on page 29). Neuronal nitric oxide synthase (nNOS) is recruited by both dystrophin itself (Lai et al., 2009) and syntrophin, which is part of the DGC (Miyagoe-Suzuki and Takeda, 2001). nNOS is activated by the increased Ca^{2+} levels during contraction, through Ca/calmodulin (Bredt and Snyder, 1990). Activation of nNOS is important for a normal muscle function, since nitric oxide (NO) relaxes smooth muscle, i.e. dilates blood vessels, preventing functional ischemia (Sander et al., 2000; Asai et al., 2007) and increasing glucose uptake (Baron et al., 1996). It also increases glucose uptake through AMPK-α-1 signaling (Deshmukh et al., 2010).

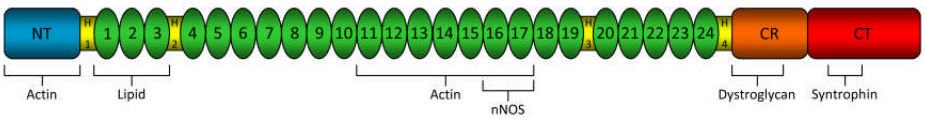


Figure 2: The dystrophin protein. The dystrophin protein can be divided in an N-terminal domain (NT), a central rod domain with spectrin-like repeats 1-24 and hinges 1-4, a cystein rich domain (CT) and a C-terminal domain (CT). Regions interacting with other molecules are depicted underneath the protein.

In DMD, pre-mature stop codons occur during transcription due to nonsense and splice-site mutations and frame shifting deletions or duplications (Aartsma-Rus et al., 2006b). The resulting proteins are truncated, no longer recruit the DGC proteins and no longer stabilize muscle fibers. As a result, muscle fibers will be damaged upon contraction and eventually will die. Symptoms in skeletal muscle and heart are most severe, although severity differs between different muscles (Porter et al., 2004). Further, dystrophin is also expressed in other tissues and the absence of dystrophin is shown to have detrimental effects in these tissues as well. For instance, a third of all patients has mental impairment, with approximately 20% having an intelligence quotient of 70 or lower (Emery, 2002). The absence of dystrophin in vascular smooth muscle cells is thought to contribute to the skeletal muscle and heart pathology (Asai et al., 2007; Loufrani et al., 2004); restoration of dystrophin only in vascular smooth muscle cells already greatly ameliorates DMD symptoms (Ito et al., 2006). Absence in the intestinal smooth muscle

cells results in slower bowel transit and lower fecal output (Mule et al., 2010). In *mdx* mice, the mouse model for DMD, prostate function is also impaired (Pinto et al., 2010).

Repair in healthy muscle

In healthy people, despite the presence of dystrophin, excessive exercise will still cause muscle damage (McNeil and Khakee, 1992). Several mechanisms are activated to repair this damage and to prevent damage in the future (see figure 3 for a simplified schematic overview, see table 1 for an overview of the main factors involved). To be able to sustain a high workload in the future, the high intracellular Ca^{2+} levels, caused by an influx of Ca^{2+} through the damaged sarcolemma and ion channels, will induce hypertrophy of the muscle fibers through the calcineurin/NFAT pathway (Jiang et al., 2009) and the insulin-like growth factor (IGF)/Akt pathway (Shavlakadze et al., 2010). To restrain hypertrophy, the IGF-1 pathway is inhibited by myostatin (Otto and Patel, 2010). Further, to repair ruptures in the sarcolemma, vesicles expressing dysferlin and trim72/mg53 are recruited within seconds after muscle damage and fuse with the sarcolemma at the site of damage (Han and Campbell, 2007; Cai et al., 2009). Trim72/mg53 also acts as a negative feedback mechanism, by inhibiting the IGF/Akt pathway (Lee et al., 2010). Finally, to remove damaged proteins, calpain 3 is activated (de Morree et al., 2010).

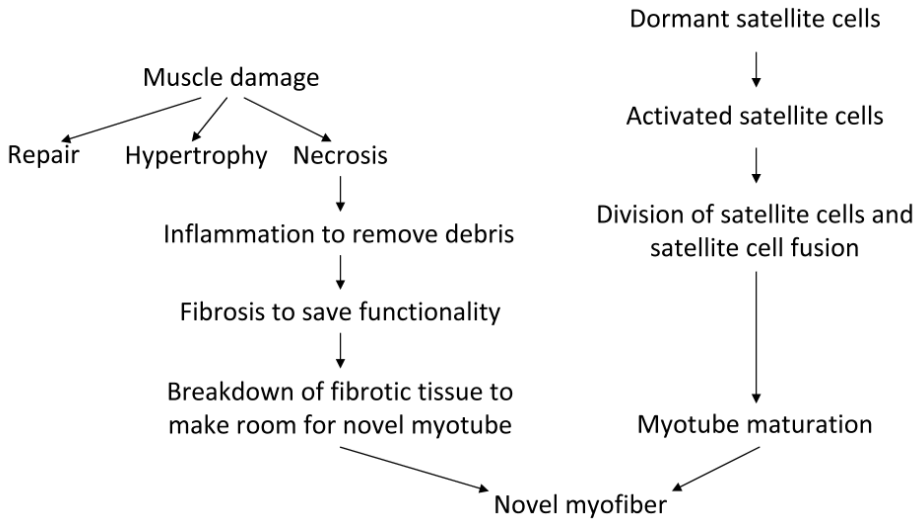


Figure 3: Simplified schematic overview of cellular mechanisms after muscle damage. In healthy muscle, repair and hypertrophy are the main mechanisms. Due to the absence of dystrophin, myofibers damage more easily in DMD and necrosis occurs much more often. Further, because of the constant cycles of necrosis and inflammation in DMD, there is not enough time for this mechanism to work properly and eventually myofibers are replaced by permanent fibrosis and fat tissue.

If part of a muscle fiber is too severely damaged to be repaired, it can be split off of the rest of the muscle fiber and be degraded (Carpenter and Karpati, 1979; Papadimitriou et al., 1990). During this process an immune reaction, including pro-inflammatory M1 macrophages, mediates clearance of the cellular debris. However, these cells also cause extra damage to the surrounding tissue and inhibit regeneration. Therefore, 2-4 days after damage the macrophage population shifts to M2 macrophages, which are anti-inflammatory and induce regeneration (Arnold et al., 2007; Tidball and Wehling-Henricks, 2007; Tidball and Villalta, 2010). TGF-β, produced by immune cells, is shown to induce this shift (Vidal et al., 2008), through induction of the IKK-β/NF-kappa-b pathway (Fong et al., 2008). On the other hand, this pathway is associated with an increase in overall inflammation (Acharyya et al., 2007) and inhibition of regeneration (Guttridge et al., 2000; Acharyya et al., 2007).

Apart from reducing inflammation and inducing regeneration, M2 macrophages furthermore induce the production of connective tissue (Vidal et al., 2008), which replaces the necrotic fibers to ensure tissue functionality and stability during regeneration (Jarvinen et al., 2005). Connective tissue is also induced by angiotensin II through up-regulation of myostatin (Li et al., 2008; Wang et al., 2008a), TGF-β (Sun et al., 2009) and osteopontin (Kupfahl et al., 2000). Interestingly, the angiotensin II type I receptor is not only activated by angiotensin II, but also by mechanical stress on the sarcolemma (Zou et al., 2004). Osteopontin does not only induce the production of connective tissue, but is shown to be important for fibroblast survival (Zohar et al., 2004) and differentiation (Lenga et al., 2008) as well. Fibroblast proliferation is induced by myostatin (Li et al., 2008) and platelet derived growth factor (PDGF), PDGF itself is up-regulated by TGF-β and the PDGF receptor β is up-regulated by angiotensin (Wang et al., 2008b). Angiotensin also reduces blood flow through vasoconstriction, which reduces oxygen supply and thereby reduces muscle activity, giving the muscle time to repair the damage. Decorin and biglycan are part of the connective tissue and are known to inhibit both myostatin and TGF-β (Brandan et al., 2008; Kishioka et al., 2008); another negative feedback mechanism, this time to prevent excessive fibrosis. Follistatin has a similar function (Abe et al., 2009b).

Table 1: Overview of the main factors involved in muscle hypertrophy, repair, degeneration and regeneration.

Factor	Effect
Angiotensin II	TGF-β up Myostatin up Platelet derived growth factor receptor up Osteopontin up Vasoconstriction VEGF up
calcineurin/NFAT pathway	Hypertrophy Interleukin-4 up
Calpain3	Remove damaged area/enable cellular remodelling
Decorin and biglycan	Inhibit myostatin Inhibit TGF-β
Dysferlin	Sarcolemma repair
Follistatin	Inhibit myostatin
Hepatocyte growth factor (HGF)	Satellite cell activation High level: myostatin
Hypoxia	MGF up Ca^{2+} (ryanodine receptor) up Myoblast differentiation
Insulin-like growth factor/Akt pathway (IGF)	Hypertrophy
Interleukin-4 (Il-4)	u-PA and u-PAR up Integrin expression on myoblasts
M1 macrophages	Increase immune reaction Cell lysis
M2 macrophages	Reduce immune reaction Induce regeneration Debris clearance Induce fibrosis Il-4 up
Mechano growth factor (MGF, IGF splice variant)	Satellite cell activation
Matrix metalloproteinases (MMPs)	Release membrane bound HGF Release membrane bound NRG1 Cleave beta-dystroglycan ECM breakdown

Myostatin	Inhibit IGF Fibroblast proliferation
Neuregulin-1	Prevent SC apoptosis Mononuclear cardiomyocytes proliferation Ca^{2+} homeostasis maintained eNOS up
Neuronal nitric oxide synthase (nNOS)	Dilated vessels/prevent ischemia Increases glucose uptake MMP up Satellite cell activation Reduce inflammation Inhibits HDAC2 → hypertrophy Upon inactivity → cytoplasm → atrophy
Notch	Satellite cell activation
Osteopontin	Fibroblast survival Fibroblast differentiation
Platelet derived growth factor (PDGF)	Fibroblast proliferation
Transforming growth factor-beta (TGF-β)	Fibrosis Platelet derived growth factor M1 macrophage → M2 macrophage
Tissue inhibitor of MMP (TIMP)	Inhibit MMP
Trim72/mg53	Sarcolemma repair Inhibit IGF/Akt
u-PA and u-PAR	plasminogen → ECM breakdown
Vascular endothelial growth factor (VEGF)	Neovasculature

To replace the necrotic myofiber that has been removed, satellite cells (SCs, the muscle stem cells) are activated by the notch pathway (Conboy et al., 2003; Turk et al., 2005), mechano growth factor (a splice variant if IGF)(Hill and Goldspink, 2003), nitric oxide (NO) and hepatocyte growth factor (HGF). In addition NO induces the production of matrix metalloproteinases (MMPs) (Hnia et al., 2007), which increase HGF levels by cleaving membrane bound HGF. Interestingly, high levels of HGF are shown to induce myostatin expression; this possibly is another negative feedback mechanism (Yamada et al., 2010).

Under influence of all the activating factors, SCs start to divide rapidly and move towards the injured area. The distance covered can be up to millimeters

(Schultz et al., 1985), but movement between different myofibers is rarely seen. SCs develop into myogenic precursor cells and fuse with each other to form a new myotube, all under the influence of the myogenic transcription factors: myoD, myf5, myogenin and mrf4 (Perry and Rudnick, 2000). These differentiating myofibers can function in the hypoxic conditions created by the angiotensin induced vasoconstriction, in fact the mouse myoblast cell line C2C12 needs hypoxia to differentiate (Ono et al., 2006). However, maturing fibers need new blood vessels to supply them with oxygen. These are formed under the influence of vascular endothelial growth factor (VEGF), which is activated by angiotensin II as well (Amaral et al., 2001). To improve satellite cell mediated repair, the damaged myofibers and the M2 macrophages produce interleukin-4 (Il-4) (Horsley et al., 2003), which has been shown to be induced by the calcineurin/NFAT pathway (Jiang et al., 2009). Il-4 up-regulates u-PA and u-PAR, which will cause ECM breakdown through plasminogen, and it up-regulates integrins, which will improve satellite cell adhesion (Lafreniere et al., 2006).

In differentiating SCs, but also in motor neurons innervating the muscle, neuregulin-1 is up-regulated (Hirata et al., 2007), for instance by cleavage of membrane bound neuregulin-1 by MMPs (Shirakabe et al., 2001; Lebrasseur et al., 2003). Neuregulin-1 prevents SCs from apoptosis (Golding et al., 2007) and is shown to induce proliferation in mononuclear cardiomyocytes (Bersell et al., 2009). Further, neuregulin-1 is shown to activate endothelial-NOS, which will enhance muscle perfusion. Finally, it is proven to have a beneficial effect on Ca^{2+} homeostasis in cardiomyocytes (Brero et al., 2010).

Like plasminogen, MMPs also break down the connective tissue and thereby make room for the newly formed myotube (Guerin and Holland, 1995; Wang et al., 2009b). To control this process, MMPs are inhibited by tissue inhibitor of metalloproteinases subtype 1 (Dennis et al., 2008). If regeneration takes too long, because there is too much damage or because regeneration is inefficient, the connective tissue will become denser and a certain amount of fibrotic scar tissue can remain (Kaariainen et al., 2000). With increasing age, satellite cells are shown to be less effective (Webster and Blau, 1990) due to the presence of this scar tissue in the interstitium (Gopinath and Rando, 2008). Further, reduced blood supply, neuromuscular junction remodeling (Gopinath and Rando, 2008) and possibly a decrease in SC number (Day et al., 2010) reduce effectiveness of SC repair. The impact of these environmental changes on functionality is underlined by the major differences seen between dystrophin knock out mice with different, otherwise healthy, genetic backgrounds (Fukada et al., 2010). Dystrophin negative mice with a mostly DBA/2 background showed to be more severely affected than standard *mdx* mice with a C57BL/10 background. This might be explained by the reduced regenerative capacity of satellite cells seen in healthy DBA/2 mice after

repeated injury (Fukada et al., 2010). Possibly elevated bone morphogenetic protein 4 (BMP4) levels cause this reduced capacity. After induction of bone formation, BMP4 levels in DBA/2 were low after 2 weeks and high after 4 weeks, while in C57BL/6, a mouse comparable to C57BL/10, the opposite was the case (Marusic et al., 1999). Together with the facts that quiescent satellite cells are shown to have high BMP4 levels (Fukada et al., 2007) and inhibition of BMP4 ameliorates symptoms in the *mdx* mouse (Shi et al., 2011), this could indicate an important role for BMP4. However, it is unknown whether satellite cells and/or other cells of DBA/2 mice also have elevated BMP4 levels after induction of muscle regeneration. Another difference is that DBA/2 mice are shown to be more prone to fibrosis, probably because of an increased activity of T helper 2 cells (Chung et al., 2003). These cells support M2 macrophages (Wehling-Henricks et al., 2010) and although M2 macrophages have beneficial effects on regeneration, they also induce fibrosis.
Finally, even in young and healthy people the regeneration process is not perfect. For instance in power-lifters, who have repeated but mild muscle damage, signs of suboptimal repair can be seen (Eriksson et al., 2006). They have split fibers, as well as an altered microstructure. Over time, such changes can lead to a decrease in force, because of altered excitation contraction coupling, and higher susceptibility to damage (Head et al., 1990; Lovering et al., 2009; Head, 2010; Friedrich et al., 2010).

Muscle damage in DMD

Due to the absence of dystrophin, myofibers of DMD patients will already be damaged after everyday use of muscles. And because of the extent and chronicity of this damage, repair mechanisms have difficulties to ensure sufficient repair. Therefore, the malformations mentioned above, which are seen in healthy subjects after many years, can already be seen in the embryo of the *mdx* mouse model (Merrick et al., 2009). A loss of satellite cells has also been seen in *mdx* (Merrick et al., 2009), however this is not seen in DMD patients (Kottlors and Kirschner, 2010). In due course, high levels of intracellular Ca^{2+}, that enter myotubes not only through the damaged sarcolemma, but also through malfunctioning transient receptor potential cation (TRPC) and stretch-activated ion channels (Bellinger et al., 2009; Fanchaouy et al., 2009; Millay et al., 2009), cause more extensive necrosis through increased mitochondrial permeability, production of reactive oxygen species (ROS) and calpain mediated protein degradation (Turner et al., 1988; Millay et al., 2008; Shkryl et al., 2009). The parts of the fibers that do survive might accumulate deletions in the mitochondrial DNA due to the reactive oxygen: mitochondrial DNA deletions accumulated in healthy aged muscle fibers are shown to lead to fiber malformation and eventually fiber loss (Herbst et al., 2007).

In healthy muscle, necrotic fibers are replaced by connective tissue, which is later mostly resolved and replaced by the regenerating myofibers. However in DMD, the degree of permanent fibrosis increases, because of the continuous damage (Chung et al., 2003). This process occurs despite anti-fibrotic mechanisms, such as down-regulation of myostatin (Chen et al., 2005) and up-regulation of decorin (Fadic et al., 2006; Abe et al., 2009a), biglycan (Fadic et al., 2006) and follistatin (Chen et al., 2005).

The constantly induced inflammation is harmful (Gosselin and McCormick, 2004; Mavrogeni et al., 2010), and also appears to be an important factor causing fibrosis, since *mdx* mice without T and B lymphocytes show a significant reduce in fibrosis (Farini et al., 2007). Possibly, the chronic inflammation and necrosis cycles even cause extra fibrosis, since fibroblasts from dystrophic muscle are shown to be constitutively active (Mezzano et al., 2007) and even satellite cells from dystrophic muscle are shown to produce collagen (Alexakis et al., 2007). If, in the future, a therapy is found which prevents damage in DMD, these fibroblasts and SCs might have to be normalized, or 'reset', to stop the pathology completely. Not only the loss and malformation of myofibers, but also fibrosis hampers muscle function, especially if it is endomysial (Desguerre et al., 2009). A close connection between SCs and endothelial cells is important for regeneration (Christov et al., 2007); this connection might be lost by increased fibrosis. Malformations of the blood vessels, such as multiple basement membranes, might also interfere with this connection (Miike et al., 1987).

As mentioned before, pathology is further increased because nNOS is not recruited to the sarcolemma (Chang et al., 1996). The absence of NO produced by nNOS will lead to functional ischemia and damage caused by functional ischemia takes longer to be repaired; force and fatigue resistance are still greatly reduced at 14 days after injury (Vignaud et al., 2010).

NO also inhibits histone deacetylase 2 (HDAC2), and an siRNA induced reduction of HDAC2 ameliorated symptoms in *mdx* muscle (Colussi et al., 2009). Class II HDAC activity is reduced upon increased Ca^{2+} levels, leading to myocyte enhancer factor 2 mediated muscle development (reviewed by McGee and Hargreaves, 2011), possibly especially of slow twitch fibers (Potthoff et al., 2007).

After a prolonged time of inactivity, nNOS is released from the DGC and moves into the cytoplasm (Suzuki et al., 2007). There it will activate the Foxo pathway, a well know pathway that leads to muscle atrophy (Sandri, 2010). It is likely that iNOS, which is also located in the cytoplasm and which has a high expression in inactive and a low expression in active muscles (Song et al., 2009), has the same effect. nNOS that is not recruited to the sarcolemma in DMD possibly has this effect as well. Furthermore, NO is shown to have an

anti-inflammatory effect (Wehling et al., 2001). Loss of NO in DMD can therefore aggravate inflammation.
Next to nNOS recruitment, α-syntrophin is also suggested to recruit voltage gated sodium channels (NaCh)(Piitulainen et al., 2008). And transient receptor potential cation channels (TRPC) are also thought to be associated with the DGC (Sabourin et al., 2009). Therefore the functionality of these channels might be impaired in DMD.

1.2 Treatment of DMD

Among the mechanisms involved in muscle repair and regeneration, there are several factors that can be interesting for DMD treatment. For instance NO donors (Mizunoya et al., 2011), since NO has a beneficial effect on all important processes, namely inflammation, fibrosis and regeneration, or neuregulin-1 up-regulation (see page 28), since it has a beneficial effect on both skeletal muscle and the heart and maybe even on the innervating neurons, or angiotensin inhibition (see page 27), since practically all fibrotic factors are increased by angiotensin. However, these are very general molecules and treatment might lead to detrimental effect elsewhere in the body. This could be overcome by specific delivery to the muscle, or by targeting a muscle specific factor in their pathways. A good example of this last option is targeting of myostatin (see page 27). Another example might be inhibition of stretch activation of the angiotensin receptor. Further, due to the complexity of repair and regeneration mechanisms, with several agents working on the same process and many negative feedback mechanisms, and additional regulation by miRNAs (Eisenberg et al., 2007; Yuasa et al., 2008; Greco et al., 2009; De, V et al., 2010), it is unlikely that changing the level of one factor will extensively normalize muscle pathology in patients. For instance, corticosteroids are shown to have a beneficial effect on several symptoms, but, although the beneficial effects are certainly noticeable, they are relatively limited (see chapter 1.2.1). If the underlying cause, absence of dystrophin, is not corrected, such treatment will only partially relieve symptoms and can only delay disease progression. Thus, treatments that can restore a (partially) functional dystrophin at the sarcolemma (see chapter 1.2.2 and 1.3) are preferred. The restored dystrophin might be sufficient for the body to adequately regulate repair and regeneration again. In case repair and regeneration still is not 100%, stimulation or inhibition of one or more factors from the repair and regeneration processes can be very helpful.

1.2.1 Current treatment

A treatment that restores dystrophin is not available yet, however, palliative treatments have proven to improve quality of life. Present day management of the disease is extensively described by Bushby and colleagues (Bushby et al., 2010a; Bushby et al., 2010b) and includes, among others, the use of assisted ventilation, corticosteroids, physical therapy, orthoses and scoliosis surgery.

Assisted ventilation
From the list of interventions, assisted ventilation has proven to be most effective. Respiratory failure used to be the main cause of death, with patients dying within a year after the first symptoms of respiratory failure. Assisted ventilation has prolonged lives up to 15 years (Eagle et al., 2002; Kohler et al., 2009) and has greatly improved quality of life as well (Dreyer et al., 2010).

Corticosteroids
In the last two decades corticosteroids like prednisone, prednisolone (the active form of prednisone) and deflazacort have also proven to be beneficial in DMD and currently they are the only medicines widely prescribed for DMD patients. Clinical trials with these corticosteroids have shown improvement in muscle strength and function, mainly on the short term, but evidence for long term effects is available as well (Manzur et al., 2008; Guerron et al., 2010). Unfortunately, long term corticosteroid treatment is accompanied by side effects, including osteoporosis, weight gain, growth inhibition, delayed puberty and cataracts, and sometimes patients cannot continue treatment because of these side effects. An alternating dosing regimen, such as 10 days on 10 days off (Straathof et al., 2009), might reduce severity of side effects, and combining corticosteroids with a synergistic drug might make it possible to lower their dose (Cozzoli et al., 2010). Also, of the used corticoids, deflazacort is thought to cause fewer side effects. Finally, the effect of corticosteroids on heart is unclear. Some studies suggest a beneficial effect (Markham et al., 2008; Mavrogeni et al., 2009), while others suggest a negative effect (Bauer et al., 2009; Guerron et al., 2010). It must be noted that the first two studies were generated from patient data, while the latter were done in mice. More research is needed to clarify these suggestions as well as the beneficial effects of corticosteroids on the long term. Currently a clinical trial, consisting of 300 patients, is being planned by NIH and TREAT-NMD to answer these questions (FOR-DMD).

The exact way in which corticosteroids alleviate symptoms is unknown, but anti-inflammatory effects are likely to be very important. The NF-κB pathway is shown to be inhibited through activation of the glucocorticoid-induced leucine zipper (GILZ) (Kagoshima et al., 2003; Ayroldi and Riccardi, 2009). Further, Wehling-Henricks and colleagues showed a decrease in immune cell specific adhesion molecules and a subsequent decrease in immune cells (Wehling-Henricks et al., 2004). Nevertheless, even in a dystrophin deficient *C. Elegans* the amount of degenerating cells is decreased after treatment with prednisone (Gaud et al., 2004). Since *C. Elegans* only has a very simple immune system this indicates that other mechanisms may be involved. This idea is supported by the fact that corticosteroids increase the tetanic force of

myofibers grown in vitro (Vandenburgh et al., 2009). No effect was seen with the anti-inflammatory drug azathioprine (Griggs et al., 1993), however the mechanism of immune inhibition is not identical for azathioprine and corticosteroids.
Corticosteroids might also increase the expression of utrophin, which can partly take over dystrophin's function (see page 26), possibly by increased transcription through the calcineurin/NF-AT pathway (St Pierre et al., 2004), though others claim utrophin mRNA levels are unaltered (Courdier-Fruh et al., 2002). Maybe utrophin protein levels are elevated by alternative translation through an internal ribosome entry site (IRES) (Miura et al., 2008). Induction of the calcineurin/NF-AT pathway also increases Il-4 expression (St Pierre et al., 2004), which can shift macrophages from the damaging M1 phenotype to the more beneficial M2 phenotype (Villalta et al., 2009), and Il-4 is expressed by differentiating muscle fibers to attract myoblasts (Horsley et al., 2003). This indicates a positive effect of corticosteroids on differentiation. On the other hand, corticosteroids are shown to down-regulate the myogenic transcription factors myogenin and myoD, through binding to the GILZ (Almon et al., 2007; Bruscoli et al., 2010).
Fibrotic agents TGF-β and collagen are also inhibited by corticosteroid treatment (Hartel et al., 2001), however this could be a result of reduced inflammation. There is evidence that corticosteroids stabilize the sarcolemma (Weissmann, 1976), act as free radical scavengers (Suzuki et al., 1985) and reduce intracellular Ca^{2+} levels (Leijendekker et al., 1996), which all would prevent most of the damage that occurs due to the lack of dystrophin. Utrophin is shown to be a substrate of the Ca^{2+} induced calpain (Courdier-Fruh and Briguet, 2006), thus the previously mentioned increase in utrophin levels might also be caused by a reduction in breakdown. Others, however, have not seen a reduction in Ca^{2+} levels after corticosteroid treatment (Vandebrouck et al., 1999).

1.2.2 Potential therapies

There is a large amount of potential therapies, aimed at alleviating a specific element of the pathology, or aimed at preventing muscle damage altogether. Efficiency of these therapies naturally depends on their target(s), but also on how much of the potential medicine is delivered to the muscle. Since approximately 40% of the body consists of muscle tissue, local injection to achieve efficient delivery is not a realistic option. In heart and diaphragm, tissues which are very important in the course of the disease, local injections are even impossible, especially when repeated injection is required.

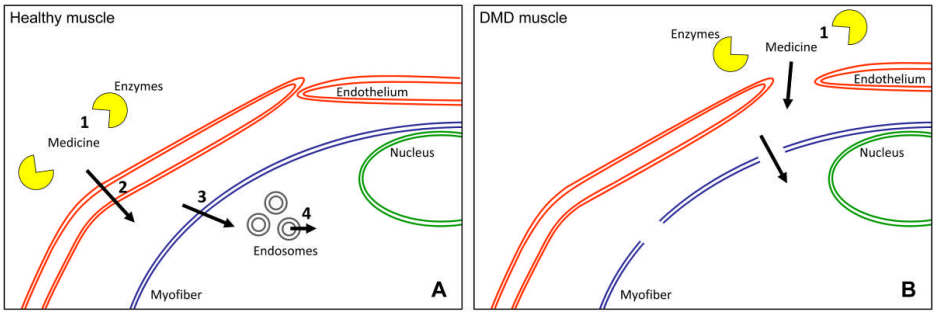


Figure 4: Barriers encountered by medicines targeting myofibers. A. In healthy muscle medicines have to: 1. withstand breakdown and alteration in the bloodstream, 2. pass the endothelium, 3. be taken up by myofibers, 4. escape from endosomes. B. In DMD, myofibers are damaged and the endothelium is less tightly closed because of the constant inflammation, therefore barriers 2, 3 and 4 are less limiting. It is unclear to what extend barriers 2, 3 and 4 will return if therapy is successful.

After systemic administration, the potential medicines have a few barriers to overcome (figure 4). First, they should not be altered or destroyed by the immune system or enzymes present in the bloodstream. Further, if they are not administered intravenously (e.g. orally, topically, subcutaneously or intraperitoneally), they should not be trapped at the delivery site, but diffuse or move to the vascular system. Next, the compound has to extravasate, preferably at the muscle tissue. For small molecules this is relatively easy, but for bigger medicines passing the endothelium is not so straightforward, some might even say it is impossible (Prof. G. Storm, personal communication). However, due to the continuous inflammation, the endothelium in DMD patients is not as tightly closed as is the case in healthy muscle. Further, vessels in DMD show malformations, which could increase permeability. On the other hand, another malformation in DMD, the increased fibrosis, is an additional barrier for the medicines. Finally, medicines have to move from the interstitium into the muscle fibers. Normally, this is a difficult barrier to cross, especially for bigger molecules. However, because of the damaged sarcolemma in DMD, medicines can move into the myofibers relatively easily (Chapter 4). If a medicine is actively taken up by the myofiber, through endocytosis, it has to escape from the endosomes as well. Finally, in case a medicine efficiently normalizes the endothelium and sarcolemma in DMD patients, these barriers will be more difficult to pass during later treatments.
Myofibers are long multinucleated fibers and it is unclear whether medicines that enter one part of the fiber also have an effect on the rest of the fiber. For instance, if a new *DMD* gene is inserted, will the mRNA or protein it produces move into the whole cell, or will it stay locally? It is known the myofiber is made up of nuclear domains, and in Drosophila it has been shown that mRNA transport is restricted to these domains (van Gemert et al., 2009). Also, shortly

after fusion of LacZ expressing human bone marrow mesenchymal stem cells to mouse myofibers, x-gal staining is confined to a small area, however, over time, the stained area increases considerably (de la Garza-Rodea AS et al., 2010). A patient with somatic mosaicism for dystrophin showed a similar effect, blood cells were 50% dystrophin positive, as expected, but muscle fibers were 80% dystrophin positive (Kesari et al., 2009). This is probably caused by replacement of dying dystrophin negative fibers by myotubes formed by dystrophin positive SCs. During regeneration, proliferating SCs move long distances along the myofibers, thus if 50% of these cells is dystrophin positive, it is possible that dystrophin will be restored in a bigger area over time. The heart does not have the same kind of regeneration as skeletal muscles, therefore this process will probably not take place in the heart (Tanaka et al., 1990). Thus, in this case pathology will be reduced in skeletal muscle but not in the heart, which could aggravate heart problems (Townsend et al., 2009). This might require additional treatment of the heart, transplantation being one of the options.
Unfortunately, cost-effectiveness is also important. Currently, the total cost of healthcare in the Netherlands is 83.8 billion euro per year, or 14.7% of the gross national product, and costs have grown approximately 6% in both 2008 and 2009 (CBS). To many people, health is much more important than money, but in countries with a mainly publicly financed healthcare system, cheaper treatments will be preferred by the government and insurance companies.

Gene therapy
In recessive genetic diseases, such as DMD, a non-mutated copy of the gene can be introduced as a treatment, using gene therapy, for instance with viral vectors. Encouraging results with integrating viral vectors have been seen in clinical trials for Leber's congenital amaurosis (Cideciyan et al., 2009) and for adrenoleukodystrophy (Cartier et al., 2009). Unfortunately, random integration in the genome can lead to oncogenesis, as seen after gene therapy of X-linked SCID patients (Hacein-Bey-Abina et al., 2008). Further, cells must divide for these viruses to integrate and myofibers are postmitotic. However, SCs are mitotic and will introduce the non-mutated gene in the myotubes by fusing with them during regeneration. Nevertheless, viruses that do not integrate their genetic code into the genome, and do not need their target cell to be mitotic, such as adeno associated viruses (AAVs), will probably be more efficient and safer. Further, DMD patients have increased fibrosis as well as less and altered capillaries. AAVs are small enough to cross this and reach the myotubes, but the other viruses can get stuck in these layers. Because they are so small, AAVs have a limited capacity and can only contain the cDNA of a drastically shortened dystrophin protein, so called micro- and minidystrophins. These dystrophins miss a large part of the central rod

domain and will not be as functional as full length dystrophin. Nevertheless, treatment is shown to induce a significant functional improvement in *mdx* and dystrophin/utrophin knock out mice (Liu et al., 2005; Wang et al., 2009a). Five months after treatment with AAV particles containing microdystrophin, 80% of the muscle fibers were dystrophin positive (Rodino-Klapac et al., 2010), but treatment was less effective in the heart (Bostick et al., 2009). Other beneficial genes might also be introduced by AAV. For instance, delivery of the myostatin propeptide, an inhibitor of myostatin, showed an increase in muscle mass and a reduction of pathology in *mdx* mice (Qiao et al., 2008). Since viruses are immunogenic, repeated treatment will be difficult and optimization is very important. So far, AAV treatment has been optimized by testing different serotypes (Wang et al., 2005), and by addition of a muscle-targeting peptide (Yu et al., 2009) (see page 37 and chapter 6 for more on muscle targeting). Accessibility can be increased by cells expressing MMP-9 and placenta growth factor (PlGF, an angiogenic factor) which are shown to reduce fibrosis and increase vascularization (Gargioli et al., 2008). A first clinical trial with local AAV injections has been conducted in the USA (Mendell et al., 2010). The treatment itself was well tolerated and micro-dystrophin was produced albeit at very low levels. However an immune reaction against this micro-dystrophin was seen in two out of six patients. This could potentially mean micro-dystrophin gene therapy is not possible in part of the DMD patients, but the significance of the immune reaction has to be further elucidated before any conclusions can be drawn. The fact that immune cells directed against dystrophin were already detected before treatment commenced, probably directed against revertant fibers, which were present despite the anti-dystrophin T-cells, might indicate the immune reaction is not very significant. Finally, although micro-dystrophin production after local injection shows some promise, local injections will not be feasible for full body treatment. Efficiency after systemic treatment will be much lower and will need a very large amount of virus, which, with current technology, will be very expensive and challenging to produce.

Cell therapy

Since skeletal muscle is repaired by fusion of stem cells (mainly the so called satellite cells) with damaged muscle fibers, introducing stem cells with a full copy of the *DMD* gene would gradually introduce dystrophin in all myofibers. Initial experiments were done with healthy donor cells, and after promising results in *mdx*/nude mice (Partridge et al., 1989), 4 out of 12 patients showed novel dystrophin in a clinical trial (Mendell et al., 1995). However, to prevent rejection, patients have to be treated with immune suppressants and these are shown to have a detrimental effect on the injected cells (Hardiman et al., 1993). As an alternative, autologous stem cells can be corrected for

dystrophin ex vivo and re-administered to the patient. Injection of un-corrected autologous muscle derived CD133+ cells showed no adverse effects, but did already show an increase in the number of capillaries per myofiber (Torrente et al., 2007). To induce a correct version of the *DMD* gene in autologous cells, there are a few options. For instance, induced pluripotent stem (iPS) cells transfected with a human artificial chromosome (HAC) produced dystrophin in chimeric mice (Kazuki et al., 2010). Muscle precursor cells (MPCs) and CD133+ cells infected with a virus coding for a u7 skipping construct (see page 35), proved to introduce dystrophin after transplantation (Quenneville et al., 2007; Benchaouir et al., 2007).

After local injection, efficiency of cell therapy is compromised by the lack of migration away from the injection site (Moens et al., 1996) and the fact a lot of the injected cells die soon after injection (Fan et al., 1996), especially if large volumes are injected (Skuk et al., 2007b). Therefore, to reach high levels of dystrophin positive fibers, up to 200 injections per square centimeter would be needed (Skuk et al., 2007a). Although such a therapy for one muscle was well tolerated, this is practically not feasible and systemic treatment would be preferred. However, the amount of cells that ends up in the muscle after systemic injection is currently very small. Although skeletal muscle is very receptive for stem cells and even metastatic cells are shown to be converted into myofibers (Parlakian et al., 2010), efficiency can still be improved by selecting the best myogenic stem cell type. Available stem cells are muscle-derived stem cells, side population cells, CD133+ cells, myoendothelial cells, perycites, mesoangioblast, multipotent adult progenitor cells, hematopoietic stem cell, mesenchymal stem cells and iPS cells (Peault et al., 2007). Another way to improve efficiency is to pre-treat cells. Cells exposed to hypoxia before treatment showed to be more efficient in a hind-limb ischemia mouse model (Leroux et al., 2010), the same is true for NO treatment (Spallotta et al., 2010). Further, combining stem cell therapy with other therapies can greatly improve efficiency, combinations with an antibody against myostatin (Geng et al., 2009), laminin-111 (Goudenege et al., 2010), the E domain of MGF (Mills et al., 2007) and cells expressing MMP-9 and PlGF (Gargioli et al., 2008) have already proven to be successful in mouse models, as well as exercise (Bouchentouf et al., 2006).

Nevertheless, although stem cell therapy has proven its potential in animal models, major improvements still have to be made to make stem cell therapy clinically applicable, especially with systemic injection. Finally, efficient expression of dystrophin in skeletal muscle has been reported to aggravate heart problems in *mdx* mice (Townsend et al., 2009), although cell therapy in the heart is showing some promise as well (Menasche, 2010).

Utrophin up-regulation

The utrophin protein is very similar to dystrophin, the N- and C-terminal domains are 80% homologous (Pearce et al., 1993) and utrophin recruits a DGC-like complex, although it does not recruit nNOS (Li et al., 2010). In developing muscle and regenerating myofibers utrophin is present along the whole sarcolemma (Galvagni et al., 2002), but in the adult muscle fiber it is restricted to the neuromuscular junction (NMJ). Notably, in DMD patients the level of utrophin is shown to correlate with disease severity (Kleopa et al., 2006), and in the *mdx* mouse, the extraocular muscles show a marked up-regulation of utrophin and do not show a dystrophic phenotype (Lewis and Ohlendieck, 2010). Artificial up-regulation of utrophin in other muscles might lead to stabilization of the myofibers. The feasibility of this approach is demonstrated by the mild phenotype seen in *mdx* mice, the severe phenotype seen in dystrophin/utrophin knock out mice (Deconinck et al., 1997) and the intermediate phenotype of the dystrophin knock out utrophin+/- mice (Zhou et al., 2008)(van Putten et al., manuscript submitted). Further, over-expression of utrophin is shown to improve the phenotype in *mdx* mice (Tinsley et al., 1996; Tinsley et al., 1998).

Compounds that increase utrophin expression have been found by screening a library of molecules. These molecules are based on known medicines and have alterations which are potentially beneficial. A first candidate compound has been tested in healthy volunteers, but serum levels detected were too low to induce utrophin up-regulation. Other candidate compounds are currently in pre-clinical testing.

Stop codon read-through

Approximately 10-15% of DMD patients have a nonsense mutation and thus a premature stop codon, while the rest of the mRNA is unaffected (Tuffery et al., 1998). A few molecules are able to read through these premature stop codons, while mostly respecting the true stop codons, these include aminoglycoside antibiotics and PTC124. PTC124 (ataluren®) has been tested in clinical trials for several diseases (Welch et al., 2007; Kerem et al., 2008) and a phase 2a clinical trial in DMD patients showed a 10% increase in dystrophin levels without inducing adverse effects in DMD. A clinical trial with a longer treatment period was performed to confirm whether the increased level of dystrophin resulted in functional improvement. Unfortunately, no improvement was seen in a 6 minutes walking test (press release: http://ptct.client.shareholder.com/releasedetail.cfm?ReleaseID=448803).

However this is a crude test and a small effect might be present. Currently, the trial is suspended and further analysis, including dystrophin analysis, is pending. Gentamicin is also shown to read through premature stop codons in

the *DMD* gene (Malik et al., 2010). Here, however, there is a risk of irreversible ototoxicity and transient nephrotoxicity upon chronic use.

Angiotensin II

Because angiotensin II induces fibrosis in DMD, inhibiting this pathway might reduce fibrosis and thereby reduce pathology. Indeed, blocking the angiotensin II receptor with losartan normalized muscle histology and function in *mdx* mice (Cohn et al., 2007). Since angiotensin II also increases angiogenesis, blocking it might reduce neovascularization and cause hypoxia in newly formed myofibers. However, this effect was not apparent in the treated *mdx* mice, thus it might be compensated by other pathways. Currently, a clinical trial with losartan is in preparation, as well as clinical trials with the angiotensin converting enzyme (ACE) inhibitors ramipril and lisinopril.

Myostatin

Myostatin inhibits muscle growth and induces fibrosis, both of which are major problems in DMD, therefore blocking myostatin is a very promising treatment option. The effects of myostatin can be blocked by reducing the levels of one of its receptors (Dumonceaux et al., 2010), increasing a myostatin inhibitor such as follistatin (Qiao et al., 2008), or by inactivating myostatin itself. The last option can be achieved with a myostatin targeting antibody. Such antibodies have been tested in patients with muscle diseases (not DMD) and were shown to be safe (Wagner et al., 2008). No increase in muscle mass or functional improvement was seen yet, but single myofibers isolated from treated patients showed improved contractile properties (Krivickas et al., 2009). Targeting the myostatin receptors might improve skeletal muscle mass more efficiently. When myostatin levels are lowered with 60%, the remaining myostatin can still influence muscle growth and fibrosis, and no beneficial effect is seen (Welle et al., 2009). If the receptors are lowered by 60%, 60% of the cells are not influenced by myostatin anymore, which, possibly, will have a greater effect. A new antibody, targeting the receptor that binds both myostatin and TGF-β, has been developed by Accelleron (recently taken over by Shire). This has shown an increase in muscle mass in healthy volunteers. Clinical trials to assess the efficacy of this antibody in Duchenne patients are currently ongoing in Canada (press release: http://www.acceleronpharma.com/content/news/pressreleases/detail.jsp/q/news-id/143). In the meantime, expressing a dominant negative myostatin led to an improvement in skeletal muscle in *mdx* mice, but did not show a significant reduction in the pathology of the diaphragm (Morine et al., 2010), especially not in older mice, which have a more advanced pathology (Murphy et al., 2010). This indicates treatment in patients should start early and that trials in older patients might have disappointing results. Further, cardiac

hypertrophy and dysfunction have been shown in *mdx* mice (Morine et al., 2010), indicating the heart is not rescued efficiently either.

Neuregulin-1
Neuregulin-1 is important for muscle regeneration and it increases utrophin expression (Basu et al., 2007). Intraperitoneal injection of a peptide encoding the epidermal growth factor-like region of neuregulin-1 showed to ameliorate the phenotype in *mdx* (Krag et al., 2004). A phase II clinical trial showed no side effects and some improvement in heart failure patients (Gao et al., 2010).

Pentoxifylline
Pentoxifylline inhibits human dermal fibroblast proliferation and the collagen synthesis by these cells (Berman and Duncan, 1989). In *mdx* no reduction in fibrosis was seen (Gosselin and Williams, 2006; Burdi et al., 2009), but Ca^{2+} homeostasis was improved and ROS were reduced (Rolland et al., 2006; Burdi et al., 2009). During a clinical trial, pentoxifylline treatment was not well tolerated by patients and it is unlikely that this compound will be developed further.

Antioxidants
A lot of the muscle damage in DMD is caused by ROS and scavenging of these could ameliorate pathology. In *mdx* mice, treatment with the antioxidant idebenone showed to be beneficial, also in the heart (Buyse et al., 2009). Clinical trials with idebenone (phase III) are ongoing. Oxandrolone, another antioxidant, is also in clinical trials.

Membrane resealing
The polymer poloxamer 188 is thought to reseal the damaged sarcolemma, but an initial test did not show a functional improvement in *mdx* mice (Quinlan et al., 2006). However, in later experiments it was shown to reduce the decline in force seen in *mdx* mice (Ng et al., 2008), and to prevent cardiac injury in the GRMD dog (Townsend et al., 2010). Poloxamer 407 is thought to improve delivery of antisense oligonucleotides (AONs, see chapter 1.3, page 29) (Lu et al., 2003; Lu et al., 2005). With poloxamer 188, we have not seen an increase in exon skipping level (Heemskerk et al, unpublished results). Possibly, the added effect of poloxamers to the exon skipping therapy is not by means of delivery but by membrane sealing.

1.3 Antisense oligonucleotide mediated exon skipping

Currently, the most promising approach to treat DMD might be antisense oligonucleotide (AON) mediated exon skipping. In this approach one or more specific exons are removed from the pre-mRNA, in order to restore the disrupted reading frame (figure 5). To achieve this exon inclusion signals, and/or splice sites are concealed from the spliceosome by AONs. As a result, the targeted exon is not recognized as such and is spliced out together with the introns. Or in other words, the exon is skipped. The resulting dystrophin protein will be shorter, but at least partially functional, since it contains the crucial C-terminal domain and can link the actin cytoskeleton to the DGC. Similar shorter proteins are found in another muscular disease, Becker muscular dystrophy (BMD). Patients with BMD are generally less severely affected than DMD patients; some are even a-symptomatic (Ferreiro et al., 2009). Incidentally, patients with spontaneous exon skipping due to a mutation in the *DMD* gene are seen (Matsuo et al., 1991; Ginjaar et al., 2000).

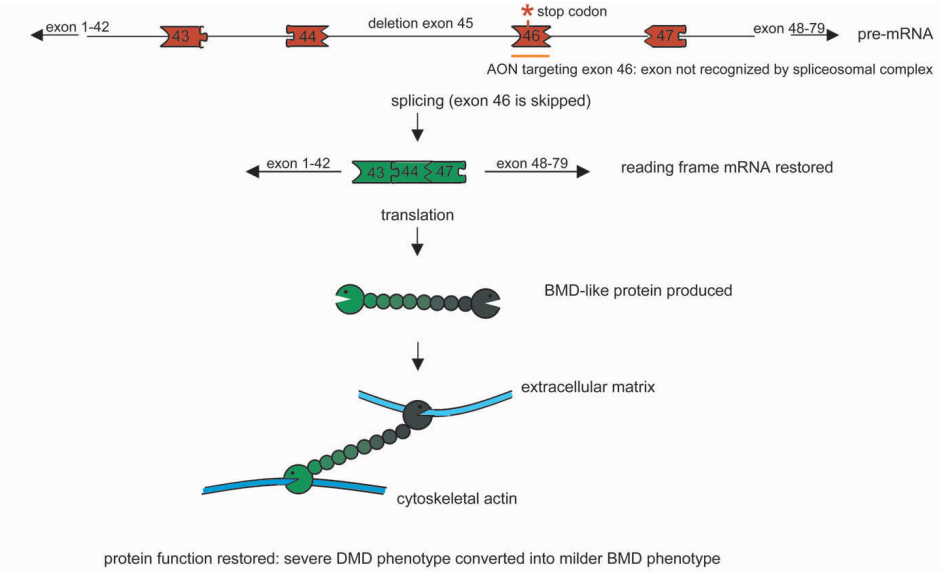


Figure 5: Schematic overview of the exon skipping process. In this example exon 45 is deleted, resulting in a premature stop codon in exon 46. When an AON targeting exon 46 is added, exon 46 is removed from the pre-mRNA, together with the introns. If the mRNA is translated a shorter but at least partially functional, BMD-like protein is produced. (Figure is kindly provided by Annemieke Aartsma-Rus)

Relatively soon after the idea was born, artificial exon skipping was induces for the first time (Pramono et al., 1996), and shortly after, the first promising exon skipping results were seen in cell culture of the *mdx* mouse (Dunckley et al., 1998; Wilton et al., 1999). In the following years, high skipping levels as

well as high protein levels were seen in cell cultures of several patients (van Deutekom et al., 2001; Aartsma-Rus et al., 2003) and after intramuscular injection in both the *mdx* mouse (Mann et al., 2001) and the hDMD mouse (Bremmer-Bout et al., 2004 and Chapter 2), which has a copy of the human *DMD* gene and facilitates testing of AONs targeting human exons in vivo. Systemic delivery of the AON also showed to be successful in the *mdx* mouse (Lu et al., 2005). Further proof of principle was shown in another animal model for DMD, the golden retriever muscular dystrophy dog (GRMD), both in vitro (McClorey et al., 2006) and systemically in vivo (Yokota et al., 2009). In the GRMD dog, exon 7 is skipped due to a mutation in intron 6, and it needs artificial skipping of exons 6 and 8 to restore the reading frame (Sharp et al., 1992). Such double exon skipping has also been successful on patient cell cultures (Aartsma-Rus et al., 2004a) and when it is successful in patients themselves as well, exon skipping will be applicable for an extra 19% of all patients (Aartsma-Rus et al., 2009). Further, it has been proposed to treat patients with a cocktail of AONs targeting a series of exons, to produce a protein that is a lot shorter than normal dystrophin, but is known to be functional (Beroud et al., 2007). Unfortunately, while single and double exon skipping have proven to be relatively straightforward, multi-exon skipping has been unsuccessful so far (Aartsma-Rus et al., 2006a; van Vliet et al., 2008). Finally, if patients miss too much of the *DMD* sequence or if they miss essential parts, it is doubtful whether they will make protein of sufficient functionality.

Meanwhile, single exon skipping has made it to the stage of clinical trials. The most promising AON chemistries available, 2'-*O*-methyl phosphorothioate (2OMePS) and phosphorodiamidate morpholino (PMO) (see page 32) have been used for these clinical trials. Local injection with 0.8 mg 2OMePS targeting exon 51 in the tibialis anterior proved to be safe and induced novel protein production in almost all myofibers in the absence of side effects (van Deutekom et al., 2007). In a parallel study, 0.09 and 0.9 mg PMO targeting exon 51 was injected in the extensor digitorum brevis. Again no adverse effects were detected and protein production was seen in most myofibers for the highest dose (Kinali et al., 2009). The 2OMePS has also been tested systemically in a phase I/II dose escalation trial, with 5 weekly subcutaneous injections of 0.5, 2, 4 and 6 mg/kg. Preliminary results show dystrophin expression in a dose dependent manner for the three higher doses (Goemans et al., manuscript accepted). The PMO has been tested with 12 intravenous injections of 0.5, 1, 2, 4, 10 and 20 mg/kg. Here preliminary results showed dystrophin expression in some of the patients in the four higher doses (Muntoni et al., presented at the World muscle society 2010, manuscript submitted). The dystrophin in these trials was clearly detectable and, although

no functional improvement is reported yet, these levels are an indication that long-term treatment might result in functional improvement as well.
The question arises how much dystrophin is needed to alleviate muscle weakness. In X-linked dilated cardiomyopathy a patient with 30% dystrophin did not have muscle weakness (Neri et al., 2007), thus 30% dystrophin might be enough. Further, a difference in age at loss of mobility was seen between patients with an average dystrophin level of 5.9% and 14.8% (Nicholson et al., 1993). As shown in figure 6, skipping levels of only 4% already reduce immunologic and fibrotic markers in *mdx* mice. This suggests that, with long-term treatment, a protein percentage of lower than 30 could have a marked effect and might even be sufficient. However, this has to be further examined and the required levels may differ for different mutations.

Despite all the promising results, research is still ongoing to further improve the efficiency of AON mediated exon skipping. One important issue to be resolved is the lower skipping efficiency in the heart (Chapter 2). High dystrophin expression in skeletal muscle and low expression in heart induces cardiomyopathy in *mdx* mice and X-linked cardiomyopathy patients (Townsend et al., 2009).

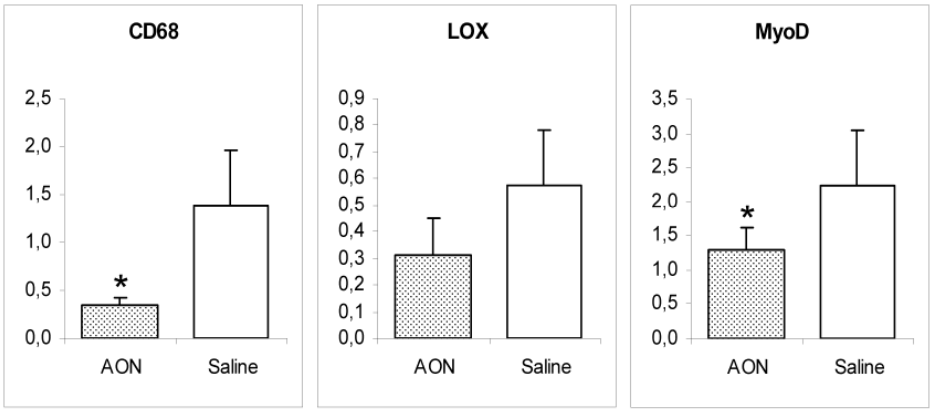


Figure 6: Low levels of exon skipping improve muscle condition. After 8 weeks of AON treatment (4 weeks 50mg/kg and 4 weeks 100mg/kg) *mdx* mice had exon skipping levels of ~4% and novel protein levels of ~1-2% (data not shown). In these mice, levels of CD68 (expressed by macrophages), Lox (involved in fibrosis) and MyoD (transcription factor increased upon regeneration) decreased. (N=4) (* $p < 0.05$)

1.3.1 Refinement of antisense oligonucleotide mediated exon skipping

Although exon skipping has already shown to be relatively efficient, refinement will make it possible to reduce the injected dose or prolong the effect. Lowering the dose will reduce the possibility of adverse effects and will make a possible therapy more cost-efficient, which means a possible therapy will become available for more patients.

Antisense oligonucleotide chemistry
To be applicable for exon skipping, AONs need to meet a few prerequisites. They should be endonuclease resistant to prevent them from being broken down, and RNAse H resistant to prevent the target RNA from being cleaved, instead of skipped. Several AON chemistries meet these prerequisites, including 2OMePS, PMO, peptide nucleic acids (PNA) and locked nucleic acids (LNA). Differences in methods make comparisons between different studies difficult (Chapter 5). Therefore, to find the optimal chemistry, they should be compared directly; however this is rarely done (Aartsma-Rus et al., 2004b; Arechavala-Gomeza et al., 2007 and Chapter 2). So far, the PMO AON has shown to be most efficient in skipping exon 23 of the *mdx* mouse (Alter et al., 2006)(Chapter 2), but for AONs inducing exon skipping in the human *DMD* gene, PMO and 2OMePS showed to be more comparable (Chapter 2). The first clinical trials with these two chemistries, using local intramuscular injections, proved to be comparable as well (van Deutekom et al., 2007; Kinali et al., 2009; Aartsma-Rus and van Ommen, 2009). Preliminary results from clinical trials with systemic administration even suggest the 2OMePS is efficient at lower doses than the PMO. The preferred AON chemistry will likely depend on the targeted sequence. An additional factor in the choice between chemistries is the sequence specificity. When AONs of the same sequence are used, PMOs have been shown to be less sequence specific (Popplewell et al., 2009 and Chapter 2). Reduced sequence specificity could result in mistargeting to other RNAs with possible detrimental effects, however so far this has never been detected.

Antisense oligonucleotide sequence
Increasing the length of an AON will increase binding affinity for the target sequence, which will increase skipping efficiency in some but not all cases (Arechavala-Gomeza et al., 2007; Harding et al., 2007 and Chapter 2). Increasing the length of an AON also increases the possibility of mistargeting. There are no definite rules for identification of the target sequence which will result in the best skipping results. However, AONs with a higher GC content and higher binding energy are significantly more effective (Aartsma-Rus et al., 2008; Popplewell et al., 2009). To optimally interfere with the splicing

machinery, sequences facilitating splicing (or 'exon inclusion signals'), e.g. exonic splice enhancer sites, seem to be the most effective targets, although AONs close to the acceptor splice site are also effective (van Ommen et al., 2000; Aartsma-Rus et al., 2008). The difference in efficiency between AONs targeting exonic splice enhancer sites and splice sites might be caused by the higher GC content in exons (Aartsma-Rus et al., 2010). The fact that AONs targeting the first half of an exon are shown to be more efficient might be related to the splicing mechanism (Wilton et al., 2007). AONs targeting sequences with a partially open configuration in the secondary structure of the RNA have been reported to be more effective as well (Aartsma-Rus et al., 2002; Wee et al., 2008; Popplewell et al., 2009).
All these analyses are based on in vitro experiments. AONs might act differently in vivo, although in vitro and in vivo results have been shown to be comparable (Wang et al., 2010). If the splicing process in the dystrophin RNA is further elucidated, more optimization of AON sequences might be possible.

Antisense oligonucleotide administration method
There are several ways in which medicines can be administered to patients. For DMD the method with the highest effect/dose ratio would be intramuscular injection. However, since approximately 40% of the body is muscle, it would be very difficult to administer AONs that way, especially because repeated treatment will be necessary. Moreover, it is impossible to repeatedly inject crucial muscles like diaphragm and heart. Systemic injection is more convenient and can be done through subcutaneous, intravenous or intraperitoneal injection. Comparison of these routes of administration, showed marked differences in uptake and exon skipping levels just after injection: intravenous shows highest immediate bioavailability followed by subcutaneous and finally intraperitoneal injection (Chapter 4). However, after intravenous injection, AON levels are also much higher in liver and kidney (Chapter 4), and high acute levels increase the risk of toxicity. Since long term availability of AON after intravenous and subcutaneous injections is comparable (Chapter 4), subcutaneous injection offers optimal characteristics, with low acute liver and kidney levels and with high availability over a longer period of time. Subcutaneous injection is also the easiest and most patient friendly method, e.g. standard insulin injection is also subcutaneous. Continuous slow subcutaneous release of AON through an implant might be even more convenient for patients. A short preliminary test with implants in *mdx* mice showed comparable results compared to subcutaneous injection (Heemskerk et al., unpublished results). Oral delivery would be the most patient friendly way of administering AONs, but it is anticipated that a lot of AON will be lost in the gastrointestinal tract.

Optimizing dosing regimen can also improve delivery. A series of low dose injections of PMOs shows an accumulation in skipping levels, which are higher than after a single high dose injection (Malerba et al., 2009). An accumulative effect is also seen with 2OMePS (Chapter 3). Research is ongoing to determine the optimal dosing regimens for both PMO and 2OMePS AONs.
Interestingly, the very pathology of DMD significantly enhances AON delivery; due to the sarcolemmal damage, myotubes of *mdx* mice take up considerably more AON than healthy myotubes (Chapter 4). Further, the constant inflammation, and possibly also the malformation of the blood vessels (Musch et al., 1975), may assist in reducing the endothelial barrier for AONs. Nevertheless, a lot of the injected dose still ends up in the kidneys and the liver (Chapter 2). Possibly, AON uptake in muscles can be further increased by massage, which has proven to work for kidney, liver and spleen (Mukai et al., 2009; Mukai et al., 2010). The increased uptake might be caused by the increase in blood flow (Mori et al., 2004) and thus mechanical loading might also improve uptake (Wu et al., 2009). Interestingly, massage is known to increase creatine kinase levels in healthy muscle (Arkko et al., 1983). This is an indicator of muscle damage, and could mean massage may aggravate pathology in DMD. However, it also means AON uptake can improve because of this damage, and if the damage is not severe and repairable, pathology might not be worsened.

Antisense oligonucleotide treatment in combination with other therapies
Ideally, AON mediated exon skipping restores expression of a sufficiently functional dystrophin to such an extent that no co-treatment is necessary. However, this might not be the case and the damage that has occurred before treatment might already have impaired functionality and the body's repair mechanisms. First of all, improving the exon skipping process itself, with chemical compounds, would increase skipping levels and thereby the efficiency of the therapy. In a screen of 2,000 compounds, guanine analogues proved to increase skipping levels (Hu et al., 2010). Other candidates for co-treatment are corticosteroids, which are currently the only medicines systematically used in the clinic (see page 20). Our comparison of AON treatment with and without prednisolone indicated an additive effect of prednisolone on muscle tissue quality (Heemskerk and Verhaart et al., manuscript submitted). However, in this pilot experiment, prednisolone treatment was given longer than AON treatment. It is possible that longer treatment with AON by itself might result in similar improvement in muscle quality. This is underlined by the finding that AON treatment resulted in dystrophin levels, which reduced the level of immunological markers to a level comparable to prednisolone treatment.

Of the potential therapies mentioned in chapter 1.2, all therapies aiming at dystrophin restoration are candidates for co-treatment with the other treatments. Of these therapies, down-regulation of myostatin has already been shown to give beneficial effects in combination with DMD exon skipping (Dumonceaux et al., 2010; Kemaladewi et al., 2011). A test in BMD patients of a myostatin antibody without AONs showed no beneficial effect (Wagner et al., 2008), while results of the myostatin receptor antibodies in DMD patients are pending. Co-treatment with a compound that stimulates satellite cell activity might be required, since satellite cells are claimed to become less active in DMD (Webster and Blau, 1990). Importantly, some of the dystrophins produced after exon skipping will not include all actin binding parts or the part that recruits nNOS (Lai et al., 2009). Since nNOS restoration without dystrophin restoration already drastically reduces pathology (Wehling et al., 2001), separate restoration of nNOS function might be necessary in these patients for an optimal result.

Finally, exon skipping can also be induced by modifying the short nuclear ribonucleoproteins U1 and U7. If the antisense units of these ribonucleoproteins are designed to mask sequences involved in splicing, the targeted exon will be skipped. Together with a promoter, these U7 or U1 genes can easily be inserted in an AAV. After delivery to the myofibers, these will produce the skip inducing U7 or U1 themselves (De Angelis et al., 2002; Goyenvalle et al., 2004; Denti et al., 2006). In itself this would be a therapy comparable with AON exon skipping, but without the burden of repeated injections. However, the same hurdles as described for gene therapy (see page 23) apply for this approach. A combination of AON and U7/U1 exon skipping could combine the best of both worlds. An initial injection with AAV containing U7 or U1 can induce exon skipping in a large number of cells. Subsequently, AON can be injected to induce skipping in all cells, and compared to treatment without AAV U7/U1, fewer injections and/or lower doses will be needed. Further, the U7 can also be inserted in the genome of mitotic cells with, for instance, a lentivirus. These can be used to correct autologous stem cells ex vivo, followed by ‘stem cell therapy’ (Benchaouir et al., 2007; Quenneville et al., 2007). This therapy can also be combined with AON mediated exon skipping. AMT is preparing a clinical trial to test a U1 small nuclear ribonucleoprotein targeting exon 51 in Duchenne patients, which is anticipated to take place in 2013.

Increasing delivery with non-viral delivery systems

A lot of research is going on in the field of non-viral oligo delivery, as can be seen in recent reviews (Mintzer and Simanek, 2009; Zhao et al., 2009; Guo et al., 2010). A lot of variations are possible and screening of non-viral delivery systems (vectors) will help in determining which vector is most efficient (Akinc

et al., 2008). Several vectors have been tested for skeletal muscle and heart delivery. Polyethylenimine (PEI) is extensively used as a transfection reagent in vitro and increases uptake and, even more important, induces endosomal escape. It has shown promise in vivo in complex with polyethylene glycol (PEG) to reduce toxicity (Sirsi et al., 2008), although PEG reduces efficiency. The recently designed poly(amido amine)s also induce endosomal escape, but are less toxic. In vitro these polymers have shown to efficiently deliver siRNA into cardiomyocytes (Kim et al., 2008). In vivo experiments are needed to assess the real potential of these polymers. Nanoparticles have already shown their potential in *mdx* mice (Ferlini et al., 2010). They prolong AON presence in the bloodstream and in the tissue. Poloxamers have a similar effect, they are inert and have been suggested to improve delivery (Lu et al., 2003; Lu et al., 2005; Yang et al., 2005), although to a lesser extent. However, the main effect of poloxamers might be resealing the damaged membrane (see page 28).

Cell penetrating peptides to improve efficiency

Cell penetrating peptides (CPPs) are short, positively charged peptides, which a-specifically bind to the cell surface. Through macropinocytosis, they are taken up by the cells (Wadia et al., 2004). Whether they escape the macropinosomes actively or passively is unknown. AONs conjugated to the HIV derived TAT protein or to the drosophila derived penetratin induce higher exon skipping levels compared to unconjugated AON in an in vitro skipping model (Turner et al., 2007). Poly(arginine) peptides are also positively charged, and therefore have similar properties. For in vivo use, it is important the poly(R) is stable both in the bloodstream and intracellularly. Adding a 6-aminohexanoic acid residue (X) and a β-alanine residue (B) increased stability in both surroundings (Youngblood et al., 2007). Several X and B containing peptides have been tested for exon skipping and for toxicity, and (RXRRBR)2XB was the optimal peptide (Wu et al., 2007; Jearawiriyapaisarn et al., 2008). Using this 'B-peptide' to deliver a PMO targeting exon 23 in *mdx* mice showed high levels of exon skipping and dystrophin protein, even in heart, in which skipping levels are normally considerably lower than skeletal muscle (Yin et al., 2008). Cardiac hypertrophy and diastolic dysfunction significantly ameliorated, up to 7 months after treatment (Jearawiriyapaisarn et al., 2010) and in the severely affected dystrophin/utrophin knock out mouse, myopathy was prevented (Goyenvalle et al., 2010). A further increase in efficiency was seen after addition of a muscle targeting peptide (Yin et al., 2009) and after addition of a lipid domain (Koppelhus et al., 2008). Because of their charge, poly(R) peptides immediately bind all cells, therefore a lot of poly(R)-AONs do not end up in the muscle. Possibly, this can be prevented by adding a polyanionic domain, which neutralizes the positive charge, through an MMP cleavable linker. This way, they can diffuse into all tissues and be

activated in tissues with high MMP levels, i.e. dystrophic muscle (Aguilera et al., 2009). A combination with a muscle homing peptide (see below) could further increase muscle specificity.
AVI-Biopharma intended to perform clinical trials with a PMO linked to the B-peptide targeting exon 50. Unfortunately, while treatment was tolerated well by mice, this compound turned out to be toxic in monkeys (Moulton and Moulton, 2010) and clinical development of the B-peptide PMO has been abandoned for now.

Phage display to find muscle homing peptides
Even when AONs have the optimal design in sequence and chemistry, and efficiency is further improved by polymers and co-treatment with other compounds, most of the injected dose will not end up in the intended organ, but in the rest of the body. In some cases this can lead to detrimental effects, as they could change the expression/form of a specific protein in an area that is not diseased. In the case of DMD, exon skipping is supposed to occur in all cells that express dystrophin, thus such detrimental effects are not anticipated. However, a lot of the AONs will end up in the kidney and the liver, which might lead to toxic effects. Furthermore, the AONs that end up in cells that do not express dystrophin are lost for skipping. If AONs are targeted to dystrophin expressing cells, the injected dose can be lowered, which will reduce the chance of adverse effects and reduce therapy cost.

The delivery of the AONs to the target tissue can be improved by homing peptides. One way to identify such peptides is by phage display (Scott and Smith, 1990; Kehoe and Kay, 2005). Different cell types express different proteins on their surface, especially diseased tissues. Peptides binding to these proteins can increase the amount of AON delivered to the target tissue. An important obstacle in this approach is the endothelium. A normally functioning endothelium will prevent entry of most compounds, and targeting peptides will not be able to bind cells in the underlying tissue. In this case peptides targeting the endothelium of the tissue of interest can be selected (Pasqualini et al., 2010). Proteins expressed on endothelium also differ between tissues, especially in a disease state, and a high local concentration of AON due to binding to the endothelium may eventually lead to higher tissue uptake, for instance in the heart (Zhang et al., 2005). The inflammation seen in DMD probably causes the endothelium to open up enough to facilitate muscle binding peptides to enter the tissue.

Phages, or more complete bacteriophages, are viruses that infect bacteria. Random peptide sequences can be added to their genome in such a way that they express these peptides on their surface (Scott and Smith, 1990; Kehoe

and Kay, 2005). This can be in a polyvalent or monovalent manner, for instance most libraries made up of the M13 phage express the random peptides on all pIII proteins (Kehoe and Kay, 2005). When a complete library of phages with random peptides is added to a molecule, cell or tissue of interest, phages expressing binding peptides can be isolated. Libraries expressing peptides of different lengths are available. Further, the attachment of the peptide to the phage can be on one side or on two sides, making the peptide circular. All these libraries contain different peptide sequences and it is important to realize that a given library will not give a complete list of all possible binding peptides. In fact, when two similar libraries with respectively 8 and 9mer peptides were compared, results did not even overlap (Kuzmicheva et al., 2008).

After selection, phages can be amplified in bacteria to allow another round of selection. More rounds of selection will increase the amount of specifically binding peptides, generally 3-5 rounds of selection are performed. However, this introduces a bias, because phages encoding some peptides replicate faster than others.
Not all peptides found with phage display will specifically bind the target sequence, in vitro and in vivo testing will be needed to confirm specificity. Before testing, websites such as Pepbank (Duchrow et al., 2009) and SAROTUP (Huang et al., 2010), can help to make a first separation. For example SAROTUP will tell you the phage expressing HAIYPRH in the Ph.D.-7 library (New England Biolabs) has a mutation that makes amplification of this phage faster (Brammer et al., 2008). These websites are only as good as the input they have, thus publishing complete lists of results will increase the usefulness of these tools. Possibly, high throughput sequencing can reduce the amount of selection rounds needed. Millions of phages can be sequenced with high throughput sequencing, reducing the risk of missing a peptide that is only modestly increased after two rounds (Di Niro et al., 2010; Dias-Neto et al., 2009 and ‘t Hoen et al., manuscript submitted). However, it is impossible to test all higher expressed peptides from such an experiment, since thousands of potentially interesting peptides are identified. Therefore data analysis and filtering is required. The previously mentioned websites are a good starting point for this, but additional criteria should be added to rank peptides.

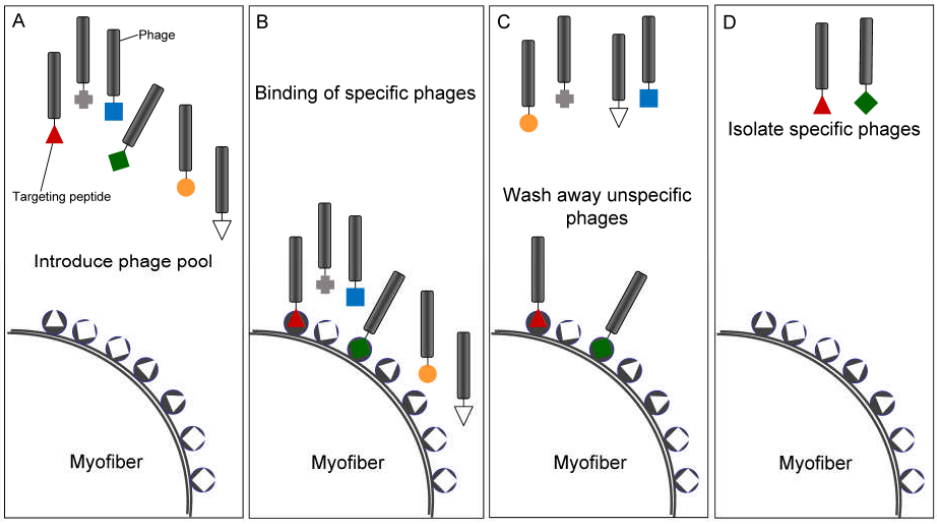


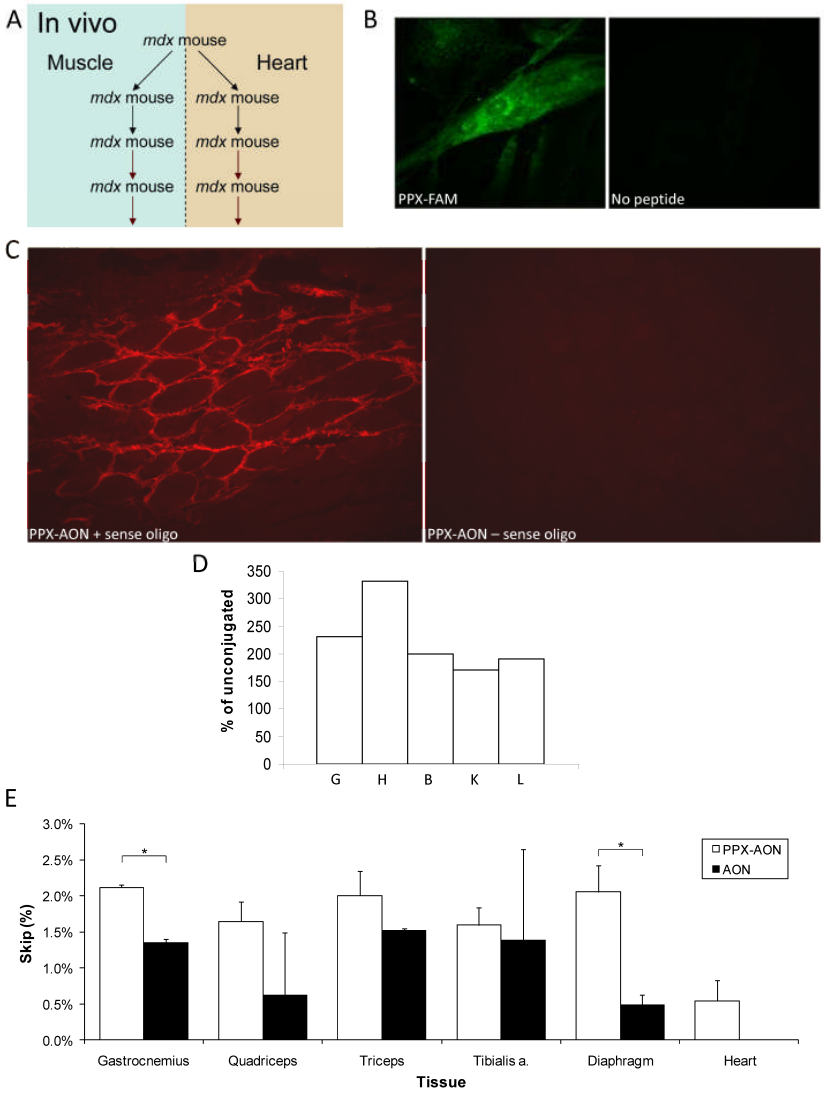
Figure 7: Schematic overview of phage display. A. A pool of phages, expressing random peptides on the outside, is added. B. Some peptides will bind, others will not. C. Unbound phages are washed away. D. Binding phages are isolated and sequenced.

Phage display can be performed on specific molecules, cells or in vivo, experiments have even been done in human subjects (Arap et al., 2002). If a molecule is proven to be highly expressed in the target tissue or the endothelium in that tissue, it is relatively easy to enrich for peptides efficiently binding to that molecule. In cell culture, the amount of potential binding sites is enormous and less enrichment will be seen. The advantage of selection on cell culture is the possibility to isolate both membrane bound phages and internalized phages. Since AONs need to enter the cells, internalized phages might be more interesting. A lot of the peptides found on muscle cells might not only bind muscle cells, but other cells as well. Also, cells in culture are known to differ considerably from cells in vivo and can express different molecules on their surface. Therefore, extensive in vivo testing of the peptides will be needed. This is also true for in vivo selection, although the comparable environment during selection and treatment can increase the success rate. Especially for in vivo selection, circulation time of the phages is important. Peptides should have enough time to bind their target, but if circulation time is too long they will be broken down. It takes about 30 minutes for a phage to be taken up by a cell (Molenaar et al., 2002) and it has been shown that the amount of isolated phages is similar after 5 and 30 minutes circulation time (Nicol et al., 2009). During a first round of in vivo selection, the most effective peptide might not be present in the isolated tissue, especially in the case of skeletal muscle, because only one of the many muscles present in the body is isolated. To make sure none of the peptides is lost, a combination of in vitro

selection followed by in vivo selection might be preferred. Finally, it is important to keep in mind that, for a given target, several targeting peptides can be found and that some of these peptides may show better results compared to AON-only treatment, but they are not the most efficient peptides.

The first muscle targeting peptide found was ASSLNIA, it was discovered by a combination of in vitro and in vivo phage display (Samoylova and Smith, 1999). When added to an AAV this peptide improved muscle delivery (Yu et al., 2009). This effect disappeared when a large amount of free peptide was co-injected, suggesting a specific targeting effect. Exon skipping levels were increased when the peptide was combined with a PMO and a CPP (Yin et al., 2009). However, while CPP-ASSLNIA-PMO showed an increase in skipping levels, ASSLNIA-CPP-PMO did not. Possibly targeting is not the primary effect in this case. A peptide targeting heart vasculature, DDTRHWG, was found by in vivo phage display (Nicol et al., 2009). The peptide proved to increase AAV delivery to the heart. Other peptides have also been shown to target muscle (Seow et al., 2010) or heart (Zahid et al., 2010). We have found a promising muscle binding peptide after performing an in vivo phage display experiment (figure 8). Of all the tested peptides, this peptide, PPX, was most promising in in vitro and intramuscular muscle binding tests. Also, after conjugation to an AON, the peptide still ensured muscle binding after intramuscular injection. Further, while 'naked' AON reaches its peak exon skipping level 10 days after intramuscular injection, a preliminary test showed PPX-AON reaches this level already after 6 hours (data not shown). This might indicate primary entrance into the cell is not through endocytosis, although the peptide clearly binds to the cell membrane, also at three days after injection. After systemic injection, more PPX-AON compared to AON was detected in all tissues, but especially in gastrocnemius muscle and heart. It should be noted that this increased level can be caused by increased binding in the tissues, but also by prolonged presence in the blood, because the blood was not removed from the tissues. Either way, these levels resulted in PPX-AON induced skipping percentages which were higher than AON induced levels. The most striking difference in exon skipping level was seen in heart and diaphragm, two tissues very important in DMD pathology. Currently, in vitro and intramuscular experiments are ongoing to shed more light on the characteristics of PPX-AON. Further, systemic experiments with higher doses are in preparation.

Figure 8: In vivo phage display experiment. An in vivo phage display experiment was performed with phages expressing 7-mer peptides as described in chapter 6. A. Subsequent rounds of phage display, performed to find heart and skeletal muscle binding peptides. Red arrows indicate phages were sequenced after those rounds of phage display. B. All peptides found more than once and peptides with 6 similar amino acids were fluorescently labeled and tested for muscle binding in vitro, peptide PPX was one of the peptides showing good muscle binding in vitro. C. Conjugated to an AON and visualized by sense staining (see chapter 6), PPX showed to ensure muscle binding after intramuscular injection. D. After systemic injection of the PPX-AON, presence in all tissues was increased, but especially in gastrocnemius muscle and heart. E. After systemic injection of the PPX-AON, skipping levels were higher compared to AON, especially in heart and diaphragm (equivalent of 50mg/kg AON, 3 injections in one week, N=2). (G = Gastrocnemius, H = Heart, B = Brain, K = Kidney, L = Liver, * = $p < 0.05$)



1.4 Concluding remarks

In the last two decades much more has happened in the field of DMD research and treatment than in the preceding 100 years following its first description. First of all, the standard use of assisted ventilation, to prevent respiratory failure, has greatly improved life for patients. Corticosteroids have also become a standard treatment, and, although the beneficial effect is not as robust as with assisted ventilation, it also shows dramatic effects.

Due to the constant damage in DMD muscle, all processes involved in repair are switched on at the same time. All these processes interfere with each other, and none of them can function as they are supposed to, resulting in severe damage and fibrosis. For treatment, all these processes can be targeted, as well as introducing a (partially) functional dystrophin to prevent damage in the first place. A lot of potential medicines have been tested, and a lot of them have shown beneficial effects in mice.

Currently, AON mediated exon skipping is the method with the most promising results in patients, and there is a reasonable chance exon skipping will become a treatment in the coming decade. However, there are a few uncertainties that have to be elucidated. The level of dystrophin needed to stabilize myofibers is unknown, and it is unknown what dystrophin levels will be feasible with exon skipping. If these levels are within the same range, exon skipping might be efficient enough on its own. If higher levels are needed, co-treatment with other medicines will be desired. Also, if the maximum dystrophin level is reached, what AON doses will be needed to sustain this level? Can doses be lowered, because only the protein turnover has to be replaced? Or do they have to be increased, because the normalized myofibers are more difficult to reach? Further, most patients will have different shorter dystrophins, and the functionality of these proteins will be different as well. Some of these proteins are comparable to proteins seen in BMD and are proven to be functional. Others will miss crucial parts of the protein and will probably be less or non-functional. And finally, for most proteins it is unknown how functional the protein will be. Ideally, pre-clinical tests should give an indication of their functionality before treatment is commenced.

Further, since patients have different mutations, different exons have to be skipped. AONs currently in clinical trials are applicable to only 20% of all patients, a number that increases to 40% if the 10 most applicable AONs are developed. Therefore, a considerable percentage of the patients cannot be treated with single AONs in the foreseeable future.

At the age of diagnosis, DMD patients already have some damage and malformation in their muscles. Possibly, this will hamper muscle function in further life, even when exon skipping proves to be very efficient. Compared to

the untreated DMD phenotype it will only be a modest effect, however, treatment should be optimized as much as possible.
Finally, therapies with more permanent restoration of a (partially) functional dystrophin, namely cell and gene therapy, might become available in the future. If this is the case, the experience that will be gained with exon skipping will be very useful.
Considering that these advances took place within 25 years of the discovery of the DMD gene and the start of functional genetics, there is good reason for optimism, both for further improvement of current approaches, and for the development of additional approaches and supporting methods.

1.5 References

Aartsma-Rus,A., Bremmer-Bout,M., Janson,A.A., den Dunnen,J.T., van Ommen,G.J., and van Deutekom,J.C. (2002). Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. Neuromuscul. Disord. *12 Suppl 1*, S71-S77.

Aartsma-Rus,A., Janson,A.A., Kaman,W.E., Bremmer-Bout,M., den Dunnen,J.T., Baas,F., van Ommen,G.J., and van Deutekom,J.C. (2003). Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum. Mol. Genet. *12*, 907-914.

Aartsma-Rus,A., Janson,A.A., Kaman,W.E., Bremmer-Bout,M., van Ommen,G.J., den Dunnen,J.T., and van Deutekom,J.C. (2004a). Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. Am. J. Hum. Genet. *74*, 83-92.

Aartsma-Rus,A., Kaman,W.E., Bremmer-Bout,M., Janson,A.A., den Dunnen,J.T., van Ommen,G.J., and van Deutekom,J.C. (2004b). Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. Gene Ther. *11*, 1391-1398.

Aartsma-Rus,A., Kaman,W.E., Weij,R., den Dunnen,J.T., van Ommen,G.J., and van Deutekom,J.C. (2006a). Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. Mol. Ther. *14*, 401-407.

Aartsma-Rus,A., van Deutekom,J.C., Fokkema,I.F., van Ommen,G.J., and den Dunnen,J.T. (2006b). Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. Muscle Nerve *34*, 135-144.

Aartsma-Rus,A., van Vliet,L., Hirschi,M., Janson,A.A., Heemskerk,H., De Winter,C.L., de Kimpe,S., van Deutekom,J.C., 't Hoen,P.A., and van Ommen,G.J. (2008). Guidelines for Antisense Oligonucleotide Design and Insight Into Splice-modulating Mechanisms. Mol. Ther. *17(3),* 548-53.

Aartsma-Rus,A., Fokkema,I., Verschuuren,J., Ginjaar,I., van Deutekom,J., van Ommen,G.J., and den Dunnen,J.T. (2009). Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. Hum. Mutat. *30*, 293-299.

Aartsma-Rus,A., Houlleberghs,H., van Deutekom,J.C., van Ommen,G.J., and 't Hoen,P.A. (2010). Exonic Sequences Provide Better Targets for Antisense Oligonucleotides Than Splice Site Sequences in the Modulation of Duchenne Muscular Dystrophy Splicing. Oligonucleotides. *20(2),* 69-77.

Aartsma-Rus,A. and van Ommen,G.J. (2009). Less is more: therapeutic exon skipping for Duchenne muscular dystrophy. Lancet Neurol. *8*, 873-875.

Abe,S., Hirose,D., Kado,S., Iwanuma,O., Saka,H., Yanagisawa,N., and Ide,Y. (2009a). Increased expression of decorin during the regeneration stage of mdx mouse. Anat. Sci. Int. *84*, 305-311.

Abe,S., Soejima,M., Iwanuma,O., Saka,H., Matsunaga,S., Sakiyama,K., and Ide,Y. (2009b). Expression of myostatin and follistatin in Mdx mice, an animal model for muscular dystrophy. Zoolog. Sci. *26*, 315-320.

Acharyya,S., Villalta,S.A., Bakkar,N., Bupha-Intr,T., Janssen,P.M., Carathers,M., Li,Z.W., Beg,A.A., Ghosh,S., Sahenk,Z., Weinstein,M., Gardner,K.L., Rafael-Fortney,J.A., Karin,M., Tidball,J.G., Baldwin,A.S., and Guttridge,D.C. (2007). Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy. J. Clin. Invest *117*, 889-901.

Aguilera,T.A., Olson,E.S., Timmers,M.M., Jiang,T., and Tsien,R.Y. (2009). Systemic in vivo distribution of activatable cell penetrating peptides is superior to that of cell penetrating peptides. Integr. Biol. (Camb.) *1*, 371-381.

Akinc,A., Zumbuehl,A., Goldberg,M., Leshchiner,E.S., Busini,V., Hossain,N., Bacallado,S.A., Nguyen,D.N., Fuller,J., Alvarez,R., Borodovsky,A., Borland,T., Constien,R., de Fougerolles,A., Dorkin,J.R., Narayanannair,J.K., Jayaraman,M., John,M., Koteliansky,V., Manoharan,M., Nechev,L., Qin,J., Racie,T., Raitcheva,D., Rajeev,K.G., Sah,D.W., Soutschek,J., Toudjarska,I., Vornlocher,H.P., Zimmermann,T.S., Langer,R., and Anderson,D.G. (2008). A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. Nat. Biotechnol. *26*, 561-569.

Alexakis,C., Partridge,T., and Bou-Gharios,G. (2007). Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction. Am. J. Physiol Cell Physiol *293*, C661-C669.

Almon,R.R., Dubois,D.C., Yao,Z., Hoffman,E.P., Ghimbovschi,S., and Jusko,W.J. (2007). Microarray analysis of the temporal response of skeletal muscle to methylprednisolone: comparative analysis of two dosing regimens. Physiol Genomics *30*, 282-299.

Alter,J., Lou,F., Rabinowitz,A., Yin,H., Rosenfeld,J., Wilton,S.D., Partridge,T.A., and Lu,Q.L. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat. Med. *12*, 175-177.

Amaral,S.L., Papanek,P.E., and Greene,A.S. (2001). Angiotensin II and VEGF are involved in angiogenesis induced by short-term exercise training. Am. J. Physiol Heart Circ. Physiol *281*, H1163-H1169.

Arap,W., Kolonin,M.G., Trepel,M., Lahdenranta,J., Cardo-Vila,M., Giordano,R.J., Mintz,P.J., Ardelt,P.U., Yao,V.J., Vidal,C.I., Chen,L., Flamm,A., Valtanen,H., Weavind,L.M., Hicks,M.E., Pollock,R.E., Botz,G.H., Bucana,C.D., Koivunen,E., Cahill,D., Troncoso,P., Baggerly,K.A., Pentz,R.D., Do,K.A., Logothetis,C.J., and Pasqualini,R. (2002). Steps toward mapping the human vasculature by phage display. Nat. Med. *8*, 121-127.

Arechavala-Gomeza,V., Graham,I.R., Popplewell,L.J., Adams,A.M., Aartsma-Rus,A., Kinali,M., Morgan,J.E., van Deutekom,J.C., Wilton,S.D., Dickson,G., and Muntoni,F. (2007). Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in human muscle. Hum. Gene Ther. *18*, 798-810.

Arkko,P.J., Pakarinen,A.J., and Kari-Koskinen,O. (1983). Effects of whole body massage on serum protein, electrolyte and hormone concentrations, enzyme activities, and hematological parameters. Int. J. Sports Med. *4*, 265-267.

Arnold,L., Henry,A., Poron,F., Baba-Amer,Y., van,R.N., Plonquet,A., Gherardi,R.K., and Chazaud,B. (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J. Exp. Med. *204*, 1057-1069.

Asai,A., Sahani,N., Kaneki,M., Ouchi,Y., Martyn,J.A., and Yasuhara,S.E. (2007). Primary role of functional ischemia, quantitative evidence for the two-hit mechanism, and phosphodiesterase-5 inhibitor therapy in mouse muscular dystrophy. PLoS. One. *2*, e806.

Ayroldi,E. and Riccardi,C. (2009). Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. FASEB J. *23*, 3649-3658.

Baron,A.D., Brechtel-Hook,G., Johnson,A., Cronin,J., Leaming,R., and Steinberg,H.O. (1996). Effect of perfusion rate on the time course of insulin-mediated skeletal muscle glucose uptake. Am. J. Physiol *271*, E1067-E1072.

Basu,U., Gyrd-Hansen,M., Baby,S.M., Lozynska,O., Krag,T.O., Jensen,C.J., Frodin,M., and Khurana,T.S. (2007). Heregulin-induced epigenetic regulation of the utrophin-A promoter. FEBS Lett. *581*, 4153-4158.

Bauer,R., Straub,V., Blain,A., Bushby,K., and MacGowan,G.A. (2009). Contrasting effects of steroids and angiotensin-converting-enzyme inhibitors in a mouse model of dystrophin-deficient cardiomyopathy. Eur. J. Heart Fail. *11*, 463-471.

Bellinger,A.M., Reiken,S., Carlson,C., Mongillo,M., Liu,X., Rothman,L., Matecki,S., Lacampagne,A., and Marks,A.R. (2009). Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. Nat. Med. *15*, 325-330.

Benchaouir,R., Meregalli,M., Farini,A., D'Antona,G., Belicchi,M., Goyenvalle,A., Battistelli,M., Bresolin,N., Bottinelli,R., Garcia,L., and Torrente,Y. (2007). Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell *1*, 646-657.

Berman,B. and Duncan,M.R. (1989). Pentoxifylline inhibits normal human dermal fibroblast in vitro proliferation, collagen, glycosaminoglycan, and fibronectin production, and increases collagenase activity. J. Invest Dermatol. *92*, 605-610.

Beroud,C., Tuffery-Giraud,S., Matsuo,M., Hamroun,D., Humbertclaude,V., Monnier,N., Moizard,M.P., Voelckel,M.A., Calemard,L.M., Boisseau,P., Blayau,M., Philippe,C., Cossee,M., Pages,M., Rivier,F., Danos,O., Garcia,L., and Claustres,M. (2007). Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. Hum. Mutat. *28(2)*, 196-202.

Bersell,K., Arab,S., Haring,B., and Kuhn,B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell *138*, 257-270.

Blake,D.J., Weir,A., Newey,S.E., and Davies,K.E. (2002). Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev. *82*, 291-329.

Bostick,B., Yue,Y., Long,C., Marschalk,N., Fine,D.M., Chen,J., and Duan,D. (2009). Cardiac expression of a mini-dystrophin that normalizes skeletal muscle force only partially restores heart function in aged Mdx mice. Mol. Ther. *17*, 253-261.

Bouchentouf,M., Benabdallah,B.F., Mills,P., and Tremblay,J.P. (2006). Exercise improves the success of myoblast transplantation in mdx mice. Neuromuscul. Disord. *16*, 518-529.

Brammer,L.A., Bolduc,B., Kass,J.L., Felice,K.M., Noren,C.J., and Hall,M.F. (2008). A target-unrelated peptide in an M13 phage display library traced to an advantageous mutation in the gene II ribosome-binding site. Anal. Biochem. *373*, 88-98.

Brandan,E., Cabello-Verrugio,C., and Vial,C. (2008). Novel regulatory mechanisms for the proteoglycans decorin and biglycan during muscle formation and muscular dystrophy. Matrix Biol. *27*, 700-708.

Bredt,D.S. and Snyder,S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U. S. A *87*, 682-685.

Bremmer-Bout,M., Aartsma-Rus,A., de Meijer,E.J., Kaman,W.E., Janson,A.A., Vossen,R.H., van Ommen,G.J., den Dunnen,J.T., and van Deutekom,J.C. (2004). Targeted exon skipping in transgenic hDMD mice: A model for direct preclinical screening of human-specific antisense oligonucleotides. Mol. Ther. *10*, 232-240.

Brero,A., Ramella,R., Fitou,A., Dati,C., Alloatti,G., Gallo,M.P., and Levi,R. (2010). Neuregulin-1{beta}1 rapidly modulates nitric oxide synthesis and calcium handling in rat cardiomyocytes. Cardiovasc. Res. *88(3),* 443-52.

Bruscoli,S., Donato,V., Velardi,E., Di Sante,M., Migliorati,G., Donato,R., and Riccardi,C. (2010). Glucocorticoid-induced leucine zipper (GILZ) and long GILZ inhibit myogenic differentiation and mediate anti-myogenic effects of glucocorticoids. J. Biol. Chem. *285*, 10385-10396.

Burdi,R., Rolland,J.F., Fraysse,B., Litvinova,K., Cozzoli,A., Giannuzzi,V., Liantonio,A., Camerino,G.M., Sblendorio,V., Capogrosso,R.F., Palmieri,B., Andreetta,F., Confalonieri,P., De Benedictis,L., Montagnani,M., and De Luca,A. (2009). Multiple pathological events in exercised dystrophic mdx mice are targeted by pentoxifylline: outcome of a large array of in vivo and ex vivo tests. J. Appl. Physiol *106*, 1311-1324.

Bushby,K., Finkel,R., Birnkrant,D.J., Case,L.E., Clemens,P.R., Cripe,L., Kaul,A., Kinnett,K., McDonald,C., Pandya,S., Poysky,J., Shapiro,F., Tomezsko,J., and Constantin,C. (2010a). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol. *9*, 77-93.

Bushby,K., Finkel,R., Birnkrant,D.J., Case,L.E., Clemens,P.R., Cripe,L., Kaul,A., Kinnett,K., McDonald,C., Pandya,S., Poysky,J., Shapiro,F., Tomezsko,J., and Constantin,C. (2010b). Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. Lancet Neurol. *9*, 177-189.

Buyse,G.M., Van der Mieren,G., Erb,M., D'hooge,J., Herijgers,P., Verbeken,E., Jara,A., Van Den Bergh,A., Mertens,L., Courdier-Fruh,I., Barzaghi,P., and Meier,T. (2009). Long-term blinded placebo-controlled study of SNT-MC17/idebenone in the dystrophin deficient mdx mouse: cardiac protection and improved exercise performance. Eur. Heart J. *30*, 116-124.

Cai,C., Masumiya,H., Weisleder,N., Matsuda,N., Nishi,M., Hwang,M., Ko,J.K., Lin,P., Thornton,A., Zhao,X., Pan,Z., Komazaki,S., Brotto,M., Takeshima,H., and Ma,J. (2009). MG53 nucleates assembly of cell membrane repair machinery. Nat. Cell Biol. *11*, 56-64.

Carpenter,S. and Karpati,G. (1979). Duchenne muscular dystrophy: plasma membrane loss initiates muscle cell necrosis unless it is repaired. Brain *102*, 147-161.

Cartier,N., Hacein-Bey-Abina,S., Bartholomae,C.C., Veres,G., Schmidt,M., Kutschera,I., Vidaud,M., Abel,U., Dal-Cortivo,L., Caccavelli,L., Mahlaoui,N., Kiermer,V., Mittelstaedt,D., Bellesme,C., Lahlou,N., Lefrere,F., Blanche,S., Audit,M., Payen,E., Leboulch,P., l'Homme,B., Bougneres,P., Von,K.C., Fischer,A., Cavazzana-Calvo,M., and Aubourg,P. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science *326*, 818-823.

Chang,W.J., Iannaccone,S.T., Lau,K.S., Masters,B.S., McCabe,T.J., McMillan,K., Padre,R.C., Spencer,M.J., Tidball,J.G., and Stull,J.T. (1996). Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy. Proc. Natl. Acad. Sci. U. S. A *93*, 9142-9147.

Chen,Y.W., Nagaraju,K., Bakay,M., McIntyre,O., Rawat,R., Shi,R., and Hoffman,E.P. (2005). Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. Neurology *65*, 826-834.

Christov,C., Chretien,F., Abou-Khalil,R., Bassez,G., Vallet,G., Authier,F.J., Bassaglia,Y., Shinin,V., Tajbakhsh,S., Chazaud,B., and Gherardi,R.K. (2007). Muscle satellite cells and endothelial cells: close neighbors and privileged partners. Mol. Biol. Cell *18*, 1397-1409.

Chung,M.P., Monick,M.M., Hamzeh,N.Y., Butler,N.S., Powers,L.S., and Hunninghake,G.W. (2003). Role of repeated lung injury and genetic background in bleomycin-induced fibrosis. Am. J. Respir. Cell Mol. Biol. *29*, 375-380.

Cideciyan,A.V., Hauswirth,W.W., Aleman,T.S., Kaushal,S., Schwartz,S.B., Boye,S.L., Windsor,E.A., Conlon,T.J., Sumaroka,A., Roman,A.J., Byrne,B.J., and Jacobson,S.G. (2009). Vision 1 year after gene therapy for Leber's congenital amaurosis. N. Engl. J Med. *361*, 725-727.

Cohn,R.D., van,E.C., Habashi,J.P., Soleimani,A.A., Klein,E.C., Lisi,M.T., Gamradt,M., ap Rhys,C.M., Holm,T.M., Loeys,B.L., Ramirez,F., Judge,D.P., Ward,C.W., and Dietz,H.C. (2007). Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Nat. Med. *13*, 204-210.

Colussi,C., Gurtner,A., Rosati,J., Illi,B., Ragone,G., Piaggio,G., Moggio,M., Lamperti,C., D'Angelo,G., Clementi,E., Minetti,G., Mozzetta,C., Antonini,A., Capogrossi,M.C., Puri,P.L., and Gaetano,C. (2009). Nitric oxide deficiency determines global chromatin changes in Duchenne muscular dystrophy. FASEB J. *23*, 2131-2141.

Conboy,I.M., Conboy,M.J., Smythe,G.M., and Rando,T.A. (2003). Notch-mediated restoration of regenerative potential to aged muscle. Science *302*, 1575-1577.

Corrado,K., Rafael,J.A., Mills,P.L., Cole,N.M., Faulkner,J.A., Wang,K., and Chamberlain,J.S. (1996). Transgenic mdx mice expressing dystrophin with a deletion in the actin-binding domain display a "mild Becker" phenotype. J. Cell Biol. *134*, 873-884.

Courdier-Fruh,I., Barman,L., Briguet,A., and Meier,T. (2002). Glucocorticoid-mediated regulation of utrophin levels in human muscle fibers. Neuromuscul. Disord. *12 Suppl 1*, S95-104.

Courdier-Fruh,I. and Briguet,A. (2006). Utrophin is a calpain substrate in muscle cells. Muscle Nerve *33*, 753-759.

Cox,G.F. and Kunkel,L.M. (1997). Dystrophies and heart disease. Curr. Opin. Cardiol. *12*, 329-343.

Cozzoli,A., Rolland,J.F., Capogrosso,R.F., Sblendorio,V.T., Longo,V., Simonetti,S., Nico,B., and De,L.A. (2010). EVALUATION OF POTENTIAL SYNERGISTIC ACTION OF A COMBINED TREATMENT WITH ALPHA-METHYL-PREDNISOLONE AND TAURINE ON THE MDX MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY. Neuropathol. Appl. Neurobiol. *37(3),* 243-56.

Day,K., Shefer,G., Shearer,A., and Yablonka-Reuveni,Z. (2010). The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. Dev. Biol. *340*, 330-343.

De Angelis,F.G., Sthandier,O., Berarducci,B., Toso,S., Galluzzi,G., Ricci,E., Cossu,G., and Bozzoni,I. (2002). Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. Proc. Natl. Acad. Sci. U. S. A *99*, 9456-9461.

de la Garza-Rodea, A.S., van,d.V., I, Boersma,H., Goncalves,M.A., van Bekkum,D.W., de Vries,A.A., and Knaan-Shanzer,S. (2010). Long-Term Contribution of Human Bone Marrow Mesenchymal Stromal Cells to Skeletal Muscle Regeneration in Mice. Cell Transplant. *20(2),* 217-31.

De,A., V, Serra,F., Cogoni,C., Vivarelli,E., Monaco,L., and Naro,F. (2010). beta1-syntrophin modulation by miR-222 in mdx mice. PLoS. One. *5*.

de Morree,M.A., Lutje,H.D., Impagliazzo,A., van Haagen,H.H., de,G.P., van,R.A., 't Hoen,P.A., van Ommen,G.B., Frants,R.R., and van der Maarel,S.M. (2010). Calpain 3 is a rapid-action, unidirectional proteolytic switch central to muscle remodeling. PLoS. One. *5*.

Deconinck,A.E., Rafael,J.A., Skinner,J.A., Brown,S.C., Potter,A.C., Metzinger,L., Watt,D.J., Dickson,J.G., Tinsley,J.M., and Davies,K.E. (1997). Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell *90*, 717-727.

Dennis,R.A., Przybyla,B., Gurley,C., Kortebein,P.M., Simpson,P., Sullivan,D.H., and Peterson,C.A. (2008). Aging alters gene expression of growth and remodeling factors in human skeletal muscle both at rest and in response to acute resistance exercise. Physiol Genomics *32*, 393-400.

Denti,M.A., Rosa,A., D'Antona,G., Sthandier,O., De Angelis,F.G., Nicoletti,C., Allocca,M., Pansarasa,O., Parente,V., Musaro,A., Auricchio,A., Bottinelli,R., and Bozzoni,I. (2006).

Body-wide gene therapy of Duchenne muscular dystrophy in the mdx mouse model. Proc. Natl. Acad. Sci. U. S. A *103*, 3758-3763.

Desguerre,I., Mayer,M., Leturcq,F., Barbet,J.P., Gherardi,R.K., and Christov,C. (2009). Endomysial fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with macrophage alternative activation. J. Neuropathol. Exp. Neurol. *68*, 762-773.

Deshmukh,A.S., Long,Y.C., de Castro,B.T., Karlsson,H.K., Glund,S., Zavadoski,W.J., Gibbs,E.M., Koistinen,H.A., Wallberg-Henriksson,H., and Zierath,J.R. (2010). Nitric oxide increases cyclic GMP levels, AMP-activated protein kinase (AMPK)alpha1-specific activity and glucose transport in human skeletal muscle. Diabetologia *53*, 1142-1150.

Di Niro,R., Sulic,A.M., Mignone,F., D'Angelo,S., Bordoni,R., Iacono,M., Marzari,R., Gaiotto,T., Lavric,M., Bradbury,A.R., Biancone,L., Zevin-Sonkin,D., De Bellis,G., Santoro,C., and Sblattero,D. (2010). Rapid interactome profiling by massive sequencing. Nucleic Acids Res. *38*, e110.

Dias-Neto,E., Nunes,D.N., Giordano,R.J., Sun,J., Botz,G.H., Yang,K., Setubal,J.C., Pasqualini,R., and Arap,W. (2009). Next-generation phage display: integrating and comparing available molecular tools to enable cost-effective high-throughput analysis. PLoS. One. *4*, e8338.

Dreyer,P.S., Steffensen,B.F., and Pedersen,B.D. (2010). Life with home mechanical ventilation for young men with Duchenne muscular dystrophy. J. Adv. Nurs. *66*, 753-762.

Duchenne, G.B.A. (1868) Recherches sur la paralysie musculaire pseudohypertrophique ou paralysie myosclérosique. Arch Gén Méd. 11, 5–25, 179–209, 305-321,421-443,552-588.

Duchrow,T., Shtatland,T., Guettler,D., Pivovarov,M., Kramer,S., and Weissleder,R. (2009). Enhancing navigation in biomedical databases by community voting and database-driven text classification. BMC. Bioinformatics. *10*, 317.

Dumonceaux,J., Marie,S., Beley,C., Trollet,C., Vignaud,A., Ferry,A., Butler-Browne,G., and Garcia,L. (2010). Combination of myostatin pathway interference and dystrophin rescue enhances tetanic and specific force in dystrophic mdx mice. Mol. Ther. *18*, 881-887.

Dunckley,M.G., Manoharan,M., Villiet,P., Eperon,I.C., and Dickson,G. (1998). Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. Hum. Mol. Genet. *7*, 1083-1090.

Eagle,M., Baudouin,S.V., Chandler,C., Giddings,D.R., Bullock,R., and Bushby,K. (2002). Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation. Neuromuscul. Disord. *12*, 926-929.

Eisenberg,I., Eran,A., Nishino,I., Moggio,M., Lamperti,C., Amato,A.A., Lidov,H.G., Kang,P.B., North,K.N., Mitrani-Rosenbaum,S., Flanigan,K.M., Neely,L.A., Whitney,D., Beggs,A.H., Kohane,I.S., and Kunkel,L.M. (2007). Distinctive patterns of microRNA expression in primary muscular disorders. Proc. Natl. Acad. Sci. U. S. A *104*, 17016-17021.

Emery,A.E. (2002). The muscular dystrophies. Lancet *359*, 687-695.

Eriksson,A., Lindstrom,M., Carlsson,L., and Thornell,L.E. (2006). Hypertrophic muscle fibers with fissures in power-lifters; fiber splitting or defect regeneration? Histochem. Cell Biol. *126*, 409-417.

Ervasti,J.M. (2007). Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. Biochim. Biophys. Acta *1772*, 108-117.

Fadic,R., Mezzano,V., Alvarez,K., Cabrera,D., Holmgren,J., and Brandan,E. (2006). Increase in decorin and biglycan in Duchenne Muscular Dystrophy: role of fibroblasts as cell source of these proteoglycans in the disease. J. Cell Mol. Med. *10*, 758-769.

Fan,Y., Maley,M., Beilharz,M., and Grounds,M. (1996). Rapid death of injected myoblasts in myoblast transfer therapy. Muscle Nerve *19*, 853-860.

Fanchaouy,M., Polakova,E., Jung,C., Ogrodnik,J., Shirokova,N., and Niggli,E. (2009). Pathways of abnormal stress-induced Ca2+ influx into dystrophic mdx cardiomyocytes. Cell Calcium *46*, 114-121.

Farini,A., Meregalli,M., Belicchi,M., Battistelli,M., Parolini,D., D'Antona,G., Gavina,M., Ottoboni,L., Constantin,G., Bottinelli,R., and Torrente,Y. (2007). T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse. J. Pathol. *213*, 229-238.

Ferlini,A., Sabatelli,P., Fabris,M., Bassi,E., Falzarano,S., Vattemi,G., Perrone,D., Gualandi,F., Maraldi,N.M., Merlini,L., Sparnacci,K., Laus,M., Caputo,A., Bonaldo,P., Braghetta,P., and Rimessi,P. (2010). Dystrophin restoration in skeletal, heart and skin arrector pili smooth muscle of mdx mice by ZM2 NP-AON complexes. Gene Ther. *17*, 432-438.

Ferreiro,V., Giliberto,F., Muniz,G.M., Francipane,L., Marzese,D.M., Mampel,A., Roque,M., Frechtel,G.D., and Szijan,I. (2009). Asymptomatic Becker muscular dystrophy in a family with a multiexon deletion. Muscle Nerve *39*, 239-243.

Fong,C.H., Bebien,M., Didierlaurent,A., Nebauer,R., Hussell,T., Broide,D., Karin,M., and Lawrence,T. (2008). An antiinflammatory role for IKKbeta through the inhibition of "classical" macrophage activation. J. Exp. Med. *205*, 1269-1276.

Friedrich,O., Both,M., Weber,C., Schurmann,S., Teichmann,M.D., von Wegner,F., Fink,R.H., Vogel,M., Chamberlain,J.S., and Garbe,C. (2010). Microarchitecture is severely compromised but motor protein function is preserved in dystrophic mdx skeletal muscle. Biophys. J. *98*, 606-616.

Fukada,S., Uezumi,A., Ikemoto,M., Masuda,S., Segawa,M., Tanimura,N., Yamamoto,H., Miyagoe-Suzuki,Y., and Takeda,S. (2007). Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells. *25(10)*, 2448-2459.

Fukada,S.I., Morikawa,D., Yamamoto,Y., Yoshida,T., Sumie,N., Yamaguchi,M., Ito,T., Miyagoe-Suzuki,Y., Takeda,S., Tsujikawa,K., and Yamamoto,H. (2010). Genetic Background Affects Properties of Satellite Cells and mdx Phenotypes. Am. J. Pathol. *176(5),* 2414-24.

Galvagni,F., Cantini,M., and Oliviero,S. (2002). The utrophin gene is transcriptionally up-regulated in regenerating muscle. J. Biol. Chem. *277*, 19106-19113.

Gao,R., Zhang,J., Cheng,L., Wu,X., Dong,W., Yang,X., Li,T., Liu,X., Xu,Y., Li,X., and Zhou,M. (2010). A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. J. Am. Coll. Cardiol. *55*, 1907-1914.

Gargioli,C., Coletta,M., De,G.F., Cannata,S.M., and Cossu,G. (2008). PlGF-MMP-9-expressing cells restore microcirculation and efficacy of cell therapy in aged dystrophic muscle. Nat. Med. *14*, 973-978.

Gaud,A., Simon,J.M., Witzel,T., Carre-Pierrat,M., Wermuth,C.G., and Segalat,L. (2004). Prednisone reduces muscle degeneration in dystrophin-deficient Caenorhabditis elegans. Neuromuscul. Disord. *14*, 365-370.

Geng,J., Peng,F., Xiong,F., Shang,Y., Zhao,C., Li,W., and Zhang,C. (2009). Inhibition of myostatin promotes myogenic differentiation of rat bone marrow-derived mesenchymal stromal cells. Cytotherapy. *11*, 849-863.

Ginjaar,I.B., Kneppers,A.L., Meulen,J.D., Anderson,L.V., Bremmer-Bout,M., van Deutekom,J.C., Weegenaar,J., den Dunnen,J.T., and Bakker,E. (2000). Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. Eur. J. Hum. Genet. *8*, 793-796.

Golding,J.P., Calderbank,E., Partridge,T.A., and Beauchamp,J.R. (2007). Skeletal muscle stem cells express anti-apoptotic ErbB receptors during activation from quiescence. Exp. Cell Res. *313*, 341-356.

Gopinath,S.D. and Rando,T.A. (2008). Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell *7*, 590-598.

Gosselin,L.E. and McCormick,K.M. (2004). Targeting the immune system to improve ventilatory function in muscular dystrophy. Med. Sci. Sports Exerc. *36*, 44-51.

Gosselin,L.E. and Williams,J.E. (2006). Pentoxifylline fails to attenuate fibrosis in dystrophic (mdx) diaphragm muscle. Muscle Nerve *33*, 820-823.

Goudenege,S., Lamarre,Y., Dumont,N., Rousseau,J., Frenette,J., Skuk,D., and Tremblay,J.P. (2010). Laminin-111: A Potential Therapeutic Agent for Duchenne Muscular Dystrophy. Mol. Ther. *18(12),* 2155-63.

Gowers W.R. (1879) Gowers, Pseudo-hypertrophic muscular paralysis: a clinical lecture. J A Churchill, London

Goyenvalle,A., Vulin,A., Fougerousse,F., Leturcq,F., Kaplan,J.C., Garcia,L., and Danos,O. (2004). Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science *306*, 1796-1799.

Goyenvalle,A., Babbs,A., Powell,D., Kole,R., Fletcher,S., Wilton,S.D., and Davies,K.E. (2010). Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. Mol. Ther. *18*, 198-205.

Greco,S., De,S.M., Colussi,C., Zaccagnini,G., Fasanaro,P., Pescatori,M., Cardani,R., Perbellini,R., Isaia,E., Sale,P., Meola,G., Capogrossi,M.C., Gaetano,C., and Martelli,F. (2009). Common micro-RNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia. FASEB J. *23*, 3335-3346.

Griggs,R.C., Moxley,R.T., III, Mendell,J.R., Fenichel,G.M., Brooke,M.H., Pestronk,A., Miller,J.P., Cwik,V.A., Pandya,S., Robison,J., and . (1993). Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). Neurology *43*, 520-527.

Guerin,C.W. and Holland,P.C. (1995). Synthesis and secretion of matrix-degrading metalloproteases by human skeletal muscle satellite cells. Dev. Dyn. *202*, 91-99.

Guerron,A.D., Rawat,R., Sali,A., Spurney,C.F., Pistilli,E., Cha,H.J., Pandey,G.S., Gernapudi,R., Francia,D., Farajian,V., Escolar,D.M., Bossi,L., Becker,M., Zerr,P., de la,P.S., Gordish-Dressman,H., Partridge,T., Hoffman,E.P., and Nagaraju,K. (2010). Functional and molecular effects of arginine butyrate and prednisone on muscle and heart in the mdx mouse model of Duchenne Muscular Dystrophy. PLoS. One. *5*, e11220.

Guo,J., Fisher,K.A., Darcy,R., Cryan,J.F., and O'Driscoll,C. (2010). Therapeutic targeting in the silent era: advances in non-viral siRNA delivery. Mol. Biosyst. *6(7),* 1143-61.

Guttridge,D.C., Mayo,M.W., Madrid,L.V., Wang,C.Y., and Baldwin,A.S., Jr. (2000). NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science *289*, 2363-2366.

Hacein-Bey-Abina,S., Garrigue,A., Wang,G.P., Soulier,J., Lim,A., Morillon,E., Clappier,E., Caccavelli,L., Delabesse,E., Beldjord,K., Asnafi,V., MacIntyre,E., Dal,C.L., Radford,I., Brousse,N., Sigaux,F., Moshous,D., Hauer,J., Borkhardt,A., Belohradsky,B.H., Wintergerst,U., Velez,M.C., Leiva,L., Sorensen,R., Wulffraat,N., Blanche,S., Bushman,F.D., Fischer,A., and Cavazzana-Calvo,M. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin. Invest *118*, 3132-3142.

Han,R. and Campbell,K.P. (2007). Dysferlin and muscle membrane repair. Curr. Opin. Cell Biol. *19*, 409-416.

Hardiman,O., Sklar,R.M., and Brown,R.H., Jr. (1993). Direct effects of cyclosporin A and cyclophosphamide on differentiation of normal human myoblasts in culture. Neurology *43*, 1432-1434.

Harding,P.L., Fall,A.M., Honeyman,K., Fletcher,S., and Wilton,S.D. (2007). The influence of antisense oligonucleotide length on dystrophin exon skipping. Mol. Ther. *15*, 157-166.

Hartel,J.V., Granchelli,J.A., Hudecki,M.S., Pollina,C.M., and Gosselin,L.E. (2001). Impact of prednisone on TGF-beta1 and collagen in diaphragm muscle from mdx mice. Muscle Nerve *24*, 428-432.

Head,S.I., Stephenson,D.G., and Williams,D.A. (1990). Properties of enzymatically isolated skeletal fibres from mice with muscular dystrophy. J. Physiol *422*, 351-367.

Head,S.I. (2010). Branched fibres in old dystrophic mdx muscle are associated with mechanical weakening of the sarcolemma, abnormal Ca2+ transients and a breakdown of Ca2+ homeostasis during fatigue. Exp. Physiol *95*, 641-656.

Herbst,A., Pak,J.W., McKenzie,D., Bua,E., Bassiouni,M., and Aiken,J.M. (2007). Accumulation of mitochondrial DNA deletion mutations in aged muscle fibers: evidence for a causal role in muscle fiber loss. J. Gerontol. A Biol. Sci. Med. Sci. *62*, 235-245.

Hill,M. and Goldspink,G. (2003). Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J. Physiol *549*, 409-418.

Hirata,M., Sakuma,K., Okajima,S., Fujiwara,H., Inashima,S., Yasuhara,M., and Kubo,T. (2007). Increased expression of neuregulin-1 in differentiating muscle satellite cells and in motoneurons during muscle regeneration. Acta Neuropathol. *113*, 451-459.

Hnia,K., Hugon,G., Rivier,F., Masmoudi,A., Mercier,J., and Mornet,D. (2007). Modulation of p38 mitogen-activated protein kinase cascade and metalloproteinase activity in diaphragm muscle in response to free radical scavenger administration in dystrophin-deficient Mdx mice. Am. J. Pathol. *170*, 633-643.

Hoffman,E.P., Brown,R.H., Jr., and Kunkel,L.M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell *51*, 919-928.

Horsley,V., Jansen,K.M., Mills,S.T., and Pavlath,G.K. (2003). IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. Cell *113*, 483-494.

Hu,Y., Wu,B., Zillmer,A., Lu,P., Benrashid,E., Wang,M., Doran,T., Shaban,M., Wu,X., and Lu,Q.L. (2010). Guanine analogues enhance antisense oligonucleotide-induced exon skipping in dystrophin gene in vitro and in vivo. Mol. Ther. *18*, 812-818.

Huang,J., Ru,B., Li,S., Lin,H., and Guo,F.B. (2010). SAROTUP: scanner and reporter of target-unrelated peptides. J. Biomed. Biotechnol. *2010*, 101932.

Ito,K., Kimura,S., Ozasa,S., Matsukura,M., Ikezawa,M., Yoshioka,K., Ueno,H., Suzuki,M., Araki,K., Yamamura,K., Miwa,T., Dickson,G., Thomas,G.D., and Miike,T. (2006). Smooth muscle-specific dystrophin expression improves aberrant vasoregulation in mdx mice. Hum. Mol. Genet. *15*, 2266-2275.

Jarvinen,T.A., Jarvinen,T.L., Kaariainen,M., Kalimo,H., and Jarvinen,M. (2005). Muscle injuries: biology and treatment. Am. J. Sports Med. *33*, 745-764.

Jearawiriyapaisarn,N., Moulton,H.M., Buckley,B., Roberts,J., Sazani,P., Fucharoen,S., Iversen,P.L., and Kole,R. (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. Mol. Ther. *16*, 1624-1629.

Jearawiriyapaisarn,N., Moulton,H.M., Sazani,P., Kole,R., and Willis,M.S. (2010). Long-term improvement in mdx cardiomyopathy after therapy with peptide-conjugated morpholino oligomers. Cardiovasc. Res. *85*, 444-453.

Jiang,L.Q., Garcia-Roves,P., de Castro,B.T., and Zierath,J.R. (2009). Constitutively active calcineurin in skeletal muscle increases endurance performance and mitochondrial respiratory capacity. Am. J Physiol Endocrinol. Metab. *298(1),* E8-E16.

Judge,L.M., Haraguchiln,M., and Chamberlain,J.S. (2006). Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex. J. Cell Sci. *119*, 1537-1546.

Kaariainen,M., Jarvinen,T., Jarvinen,M., Rantanen,J., and Kalimo,H. (2000). Relation between myofibers and connective tissue during muscle injury repair. Scand. J. Med. Sci. Sports *10*, 332-337.

Kagoshima,M., Ito,K., Cosio,B., and Adcock,I.M. (2003). Glucocorticoid suppression of nuclear factor-kappa B: a role for histone modifications. Biochem. Soc. Trans. *31*, 60-65.

Kazuki,Y., Hiratsuka,M., Takiguchi,M., Osaki,M., Kajitani,N., Hoshiya,H., Hiramatsu,K., Yoshino,T., Kazuki,K., Ishihara,C., Takehara,S., Higaki,K., Nakagawa,M., Takahashi,K., Yamanaka,S., and Oshimura,M. (2010). Complete genetic correction of ips cells from Duchenne muscular dystrophy. Mol. Ther. *18*, 386-393.

Kehoe,J.W. and Kay,B.K. (2005). Filamentous phage display in the new millennium. Chem. Rev. *105*, 4056-4072.

Kemaladewi,D.U., Hoogaars,W.M., van Heiningen,S.H., Terlouw,S., de Gorter,D.J., den Dunnen,J.T., van Ommen,G.J., Aartsma-Rus,A., Ten,D.P., and 't Hoen,P.A. (2011). Dual exon skipping in myostatin and dystrophin for Duchenne muscular dystrophy. BMC. Med. Genomics. *20;4*, 36.

Kerem,E., Hirawat,S., Armoni,S., Yaakov,Y., Shoseyov,D., Cohen,M., Nissim-Rafinia,M., Blau,H., Rivlin,J., Aviram,M., Elfring,G.L., Northcutt,V.J., Miller,L.L., Kerem,B., and Wilschanski,M. (2008). Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. Lancet *372*, 719-727.

Kesari,A., Neel,R., Wagoner,L., Harmon,B., Spurney,C., and Hoffman,E.P. (2009). Somatic mosaicism for Duchenne dystrophy: evidence for genetic normalization mitigating muscle symptoms. Am. J. Med. Genet. A *149A*, 1499-1503.

Kim,S.H., Jeong,J.H., Ou,M., Yockman,J.W., Kim,S.W., and Bull,D.A. (2008). Cardiomyocyte-targeted siRNA delivery by prostaglandin E(2)-Fas siRNA polyplexes formulated with reducible poly(amido amine) for preventing cardiomyocyte apoptosis. Biomaterials *29*, 4439-4446.

Kinali,M., Arechavala-Gomeza,V., Feng,L., Cirak,S., Hunt,D., Adkin,C., Guglieri,M., Ashton,E., Abbs,S., Nihoyannopoulos,P., Garralda,M.E., Rutherford,M., McCulley,C., Popplewell,L., Graham,I.R., Dickson,G., Wood,M.J., Wells,D.J., Wilton,S.D., Kole,R., Straub,V., Bushby,K., Sewry,C., Morgan,J.E., and Muntoni,F. (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol. *8*, 918-928.

Kishioka,Y., Thomas,M., Wakamatsu,J., Hattori,A., Sharma,M., Kambadur,R., and Nishimura,T. (2008). Decorin enhances the proliferation and differentiation of myogenic cells through suppressing myostatin activity. J. Cell Physiol *215*, 856-867.

Kleopa,K.A., Drousiotou,A., Mavrikiou,E., Ormiston,A., and Kyriakides,T. (2006). Naturally occurring utrophin correlates with disease severity in Duchenne muscular dystrophy. Hum. Mol. Genet. *15*, 1623-1628.

Koenig,M., Hoffman,E.P., Bertelson,C.J., Monaco,A.P., Feener,C., and Kunkel,L.M. (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell *50*, 509-517.

Kohler,M., Clarenbach,C.F., Bahler,C., Brack,T., Russi,E.W., and Bloch,K.E. (2009). Disability and survival in Duchenne muscular dystrophy. J. Neurol. Neurosurg. Psychiatry *80*, 320-325.

Koppelhus,U., Shiraishi,T., Zachar,V., Pankratova,S., and Nielsen,P.E. (2008). Improved cellular activity of antisense peptide nucleic acids by conjugation to a cationic peptide-lipid (CatLip) domain. Bioconjug. Chem. *19*, 1526-1534.

Kottlors,M. and Kirschner,J. (2010). Elevated satellite cell number in Duchenne muscular dystrophy. Cell Tissue Res. *340(3),* 541-8.

Krag,T.O., Bogdanovich,S., Jensen,C.J., Fischer,M.D., Hansen-Schwartz,J., Javazon,E.H., Flake,A.W., Edvinsson,L., and Khurana,T.S. (2004). Heregulin ameliorates the dystrophic phenotype in mdx mice. Proc. Natl. Acad. Sci. U. S. A *101*, 13856-13860.

Krivickas,L.S., Walsh,R., and Amato,A.A. (2009). Single muscle fiber contractile properties in adults with muscular dystrophy treated with MYO-029. Muscle Nerve *39*, 3-9.

Kupfahl,C., Pink,D., Friedrich,K., Zurbrugg,H.R., Neuss,M., Warnecke,C., Fielitz,J., Graf,K., Fleck,E., and Regitz-Zagrosek,V. (2000). Angiotensin II directly increases transforming growth factor beta1 and osteopontin and indirectly affects collagen mRNA expression in the human heart. Cardiovasc. Res. *46*, 463-475.

Kuzmicheva,G.A., Jayanna,P.K., Sorokulova,I.B., and Petrenko,V.A. (2008). Diversity and censoring of landscape phage libraries. Protein Eng Des Sel. *22(1),* 9-18.

Lafreniere,J.F., Mills,P., Bouchentouf,M., and Tremblay,J.P. (2006). Interleukin-4 improves the migration of human myogenic precursor cells in vitro and in vivo. Exp. Cell Res. *312*, 1127-1141.

Lai,Y., Thomas,G.D., Yue,Y., Yang,H.T., Li,D., Long,C., Judge,L., Bostick,B., Chamberlain,J.S., Terjung,R.L., and Duan,D. (2009). Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. J. Clin. Invest *119*, 624-635.

Lebrasseur,N.K., Cote,G.M., Miller,T.A., Fielding,R.A., and Sawyer,D.B. (2003). Regulation of neuregulin/ErbB signaling by contractile activity in skeletal muscle. Am. J. Physiol Cell Physiol *284*, C1149-C1155.

Lee,C.S., Yi,J.S., Jung,S.Y., Kim,B.W., Lee,N.R., Choo,H.J., Jang,S.Y., Han,J., Chi,S.G., Park,M., Lee,J.H., and Ko,Y.G. (2010). TRIM72 negatively regulates myogenesis via targeting insulin receptor substrate-1. Cell Death. Differ. *17*, 1254-1265.

Legardinier,S., Hubert,J.F., Le,B.O., Tascon,C., Rocher,C., Raguenes-Nicol,C., Bondon,A., Hardy,S., and Le,R.E. (2008). Sub-domains of the dystrophin rod domain display contrasting lipid-binding and stability properties. Biochim. Biophys. Acta *1784*, 672-682.

Leijendekker,W.J., Passaquin,A.C., Metzinger,L., and Ruegg,U.T. (1996). Regulation of cytosolic calcium in skeletal muscle cells of the mdx mouse under conditions of stress. Br. J. Pharmacol. *118*, 611-616.

Lenga,Y., Koh,A., Perera,A.S., McCulloch,C.A., Sodek,J., and Zohar,R. (2008). Osteopontin expression is required for myofibroblast differentiation. Circ. Res. *102*, 319-327.

Leroux,L., Descamps,B., Tojais,N.F., Seguy,B., Oses,P., Moreau,C., Daret,D., Ivanovic,Z., Boiron,J.M., Lamaziere,J.M., Dufourcq,P., Couffinhal,T., and Duplaa,C. (2010). Hypoxia Preconditioned Mesenchymal Stem Cells Improve Vascular and Skeletal Muscle Fiber Regeneration After Ischemia Through a Wnt4-dependent Pathway. Mol. Ther. *18*, 1545-1552.

Lewis,C. and Ohlendieck,K. (2010). Proteomic profiling of naturally protected extraocular muscles from the dystrophin-deficient mdx mouse. Biochem. Biophys. Res. Commun. *396*, 1024-1029.

Li,D., Bareja,A., Judge,L., Yue,Y., Lai,Y., Fairclough,R., Davies,K.E., Chamberlain,J.S., and Duan,D. (2010). Sarcolemmal nNOS anchoring reveals a qualitative difference between dystrophin and utrophin. J. Cell Sci. *123*, 2008-2013.

Liu,M., Yue,Y., Harper,S.Q., Grange,R.W., Chamberlain,J.S., and Duan,D. (2005). Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury. Mol. Ther. *11*, 245-256.

Li,Z.B., Kollias,H.D., and Wagner,K.R. (2008). Myostatin directly regulates skeletal muscle fibrosis. J Biol. Chem. *283*, 19371-19378.

Loufrani,L., Dubroca,C., You,D., Li,Z., Levy,B., Paulin,D., and Henrion,D. (2004). Absence of dystrophin in mice reduces NO-dependent vascular function and vascular density: total recovery after a treatment with the aminoglycoside gentamicin. Arterioscler. Thromb. Vasc. Biol. *24*, 671-676.

Lovering,R.M., Michaelson,L., and Ward,C.W. (2009). Malformed mdx myofibers have normal cytoskeletal architecture yet altered EC coupling and stress-induced Ca2+ signaling. Am. J. Physiol Cell Physiol *297*, C571-C580.

Lu,Q.L., Mann,C.J., Lou,F., Bou-Gharios,G., Morris,G.E., Xue,S.A., Fletcher,S., Partridge,T.A., and Wilton,S.D. (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat. Med. *9*, 1009-1014.

Lu,Q.L., Rabinowitz,A., Chen,Y.C., Yokota,T., Yin,H., Alter,J., Jadoon,A., Bou-Gharios,G., and Partridge,T. (2005). Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc Natl Acad Sci U S A *102*, 198-203.

Malerba,A., Thorogood,F.C., Dickson,G., and Graham,I.R. (2009). Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice. Hum. Gene Ther. *20*, 955-965.

Malik,V., Rodino-Klapac,L.R., Viollet,L., Wall,C., King,W., Al-Dahhak,R., Lewis,S., Shilling,C.J., Kota,J., Serrano-Munuera,C., Hayes,J., Mahan,J.D., Campbell,K.J., Banwell,B., Dasouki,M., Watts,V., Sivakumar,K., Bien-Willner,R., Flanigan,K.M., Sahenk,Z., Barohn,R.J., Walker,C.M., and Mendell,J.R. (2010). Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy. Ann. Neurol. *67*, 771-780.

Mann,C.J., Honeyman,K., Cheng,A.J., Ly,T., Lloyd,F., Fletcher,S., Morgan,J.E., Partridge,T.A., and Wilton,S.D. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. Proc. Natl. Acad. Sci U. S. A *98*, 42-47.

Manzur,A.Y., Kuntzer,T., Pike,M., and Swan,A. (2008). Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane. Database. Syst. Rev. CD003725.

Markham,L.W., Kinnett,K., Wong,B.L., Woodrow,B.D., and Cripe,L.H. (2008). Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy. Neuromuscul. Disord. *18*, 365-370.

Marusic,A., Katavic,V., Grcevic,D., and Lukic,I.K. (1999). Genetic variability of new bone induction in mice. Bone. *25(1)*, 25-32.

Matsuo,M., Masumura,T., Nishio,H., Nakajima,T., Kitoh,Y., Takumi,T., Koga,J., and Nakamura,H. (1991). Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy kobe. J Clin. Invest *87*, 2127-2131.

Mavrogeni,S., Papavasiliou,A., Douskou,M., Kolovou,G., Papadopoulou,E., and Cokkinos,D.V. (2009). Effect of deflazacort on cardiac and sternocleidomastoid muscles in Duchenne muscular dystrophy: a magnetic resonance imaging study. Eur. J. Paediatr. Neurol. *13*, 34-40.

Mavrogeni,S., Papavasiliou,A., Spargias,K., Constandoulakis,P., Papadopoulos,G., Karanasios,E., Georgakopoulos,D., Kolovou,G., Demerouti,E., Polymeros,S., Kaklamanis,L., Magoutas,A., Papadopoulou,E., Markussis,V., and Cokkinos,D.V. (2010). Myocardial inflammation in Duchenne Muscular Dystrophy as a precipitating factor for heart failure: a prospective study. BMC. Neurol. *10*, 33.

McClorey,G., Moulton,H.M., Iversen,P.L., Fletcher,S., and Wilton,S.D. (2006). Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. Gene Ther. *13*, 1373-1381.

McGee,S.L. and Hargreaves,M. (2011). Histone modifications and exercise adaptations. J. Appl. Physiol. *110(1)*, 258-263.

McNeil,P.L. and Khakee,R. (1992). Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. Am. J. Pathol. *140*, 1097-1109.

Menasche,P. (2010). Cardiac cell therapy: Lessons from clinical trials. J. Mol. Cell Cardiol. *50(2),* 258-65.

Mendell,J.R., Kissel,J.T., Amato,A.A., King,W., Signore,L., Prior,T.W., Sahenk,Z., Benson,S., McAndrew,P.E., Rice,R., and . (1995). Myoblast transfer in the treatment of Duchenne's muscular dystrophy. N. Engl. J Med. *333*, 832-838.

Mendell,J.R., Campbell,K., Rodino-Klapac,L., Sahenk,Z., Shilling,C., Lewis,S., Bowles,D., Gray,S., Li,C., Galloway,G., Malik,V., Coley,B., Clark,K.R., Li,J., Xiao,X., Samulski,J., McPhee,S.W., Samulski,R.J., and Walker,C.M. (2010). Dystrophin immunity in Duchenne's muscular dystrophy. N. Engl. J. Med. *363(15)*, 1429-1437.

Merrick,D., Stadler,L.K., Larner,D., and Smith,J. (2009). Muscular dystrophy begins early in embryonic development deriving from stem cell loss and disrupted skeletal muscle formation. Dis. Model. Mech. *2*, 374-388.

Meryon, E. (1852). On granular and fatty degeneration of the voluntary muscles. Med Chir Trans. *35,* 73–84.

Mezzano,V., Cabrera,D., Vial,C., and Brandan,E. (2007). Constitutively activated dystrophic muscle fibroblasts show a paradoxical response to TGF-beta and CTGF/CCN2. J. Cell Commun. Signal. *1*, 205-217.

Miike,T., Sugino,S., Ohtani,Y., Taku,K., and Yoshioka,K. (1987). Vascular endothelial cell injury and platelet embolism in Duchenne muscular dystrophy at the preclinical stage. J. Neurol. Sci. *82*, 67-80.

Millay,D.P., Sargent,M.A., Osinska,H., Baines,C.P., Barton,E.R., Vuagniaux,G., Sweeney,H.L., Robbins,J., and Molkentin,J.D. (2008). Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. Nat. Med. *14*, 442-447.

Millay,D.P., Goonasekera,S.A., Sargent,M.A., Maillet,M., Aronow,B.J., and Molkentin,J.D. (2009). Calcium influx is sufficient to induce muscular dystrophy through a TRPC-dependent mechanism. Proc. Natl. Acad. Sci. U. S. A *106*, 19023-19028.

Mills,P., Lafreniere,J.F., Benabdallah,B.F., El Fahime,e.M., and Tremblay,J.P. (2007). A new pro-migratory activity on human myogenic precursor cells for a synthetic peptide within the E domain of the mechano growth factor. Exp. Cell Res. *313*, 527-537.

Mintzer,M.A. and Simanek,E.E. (2009). Nonviral vectors for gene delivery. Chem. Rev. *109*, 259-302.

Mirza,A., Sagathevan,M., Sahni,N., Choi,L., and Menhart,N. (2010). A biophysical map of the dystrophin rod. Biochim. Biophys. Acta. *1804(9),* 1796-809.

Miura,P., Andrews,M., Holcik,M., and Jasmin,B.J. (2008). IRES-mediated translation of utrophin A is enhanced by glucocorticoid treatment in skeletal muscle cells. PLoS. One. *3*, e2309.

Miyagoe-Suzuki,Y. and Takeda,S.I. (2001). Association of neuronal nitric oxide synthase (nNOS) with alpha1-syntrophin at the sarcolemma. Microsc. Res. Tech. *55*, 164-170.

Mizunoya,W., Upadhaya,R., Burczynski,F.J., Wang,G., and Anderson,J.E. (2011). Nitric-oxide donors improve prednisone effects on muscular dystrophy in the mdx mouse diaphragm. Am. J. Physiol Cell Physiol. *300(5),* C1065-77.

Moens,P.D., Van-Schoor,M.C., and Marechal,G. (1996). Lack of myoblasts migration between transplanted and host muscles of mdx and normal mice. J. Muscle Res. Cell Motil. *17*, 37-43.

Molenaar,T.J., Michon,I., de Haas,S.A., Van Berkel,T.J., Kuiper,J., and Biessen,E.A. (2002). Uptake and processing of modified bacteriophage M13 in mice: implications for phage display. Virology *293*, 182-191.

Mori,H., Ohsawa,H., Tanaka,T.H., Taniwaki,E., Leisman,G., and Nishijo,K. (2004). Effect of massage on blood flow and muscle fatigue following isometric lumbar exercise. Med. Sci. Monit. *10*, CR173-CR178.

Morine,K.J., Bish,L.T., Pendrak,K., Sleeper,M.M., Barton,E.R., and Sweeney,H.L. (2010). Systemic myostatin inhibition via liver-targeted gene transfer in normal and dystrophic mice. PLoS. One. *5*, e9176.

Moulton,H.M. and Moulton,J.D. (2010). Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy. Biochim. Biophys. Acta. *1798(12),* 2296-303.

Mukai,H., Kawakami,S., Kamiya,Y., Ma,F., Takahashi,H., Satake,K., Terao,K., Kotera,H., Yamashita,F., and Hashida,M. (2009). Pressure-mediated transfection of murine spleen and liver. Hum. Gene Ther. *20*, 1157-1167.

Mukai,H., Kawakami,S., Takahashi,H., Satake,K., Yamashita,F., and Hashida,M. (2010). Key physiological phenomena governing transgene expression based on tissue pressure-mediated transfection in mice. Biol. Pharm. Bull. *33*, 1627-1632.

Mule,F., Amato,A., and Serio,R. (2010). Gastric emptying, small intestinal transit and fecal output in dystrophic (mdx) mice. J. Physiol Sci. *60*, 75-79.

Murphy,K.T., Ryall,J.G., Snell,S.M., Nair,L., Koopman,R., Krasney,P.A., Ibebunjo,C., Holden,K.S., Loria,P.M., Salatto,C.T., and Lynch,G.S. (2010). Antibody-Directed Myostatin Inhibition Improves Diaphragm Pathology in Young but not Adult Dystrophic mdx Mice. Am. J. Pathol. *176(5),* 2425-34.

Musch,B.C., Papapetropoulos,T.A., McQueen,D.A., Hudgson,P., and Weightman,D. (1975). A comparison of the structure of small blood vessels in normal, denervated and dystrophic human muscle. J. Neurol. Sci. *26*, 221-234.

Neri,M., Torelli,S., Brown,S., Ugo,I., Sabatelli,P., Merlini,L., Spitali,P., Rimessi,P., Gualandi,F., Sewry,C., Ferlini,A., and Muntoni,F. (2007). Dystrophin levels as low as 30% are sufficient to avoid muscular dystrophy in the human. Neuromuscul. Disord. *17*, 913-918.

Ng,R., Metzger,J.M., Claflin,D.R., and Faulkner,J.A. (2008). Poloxamer 188 reduces the contraction-induced force decline in lumbrical muscles from mdx mice. Am. J. Physiol Cell Physiol *295*, C146-C150.

Nicholson,L.V., Johnson,M.A., Bushby,K.M., and Gardner-Medwin,D. (1993). Functional significance of dystrophin positive fibres in Duchenne muscular dystrophy. Arch. Dis. Child *68*, 632-636.

Nicol,C.G., Denby,L., Lopez-Franco,O., Masson,R., Halliday,C.A., Nicklin,S.A., Kritz,A., Work,L.M., and Baker,A.H. (2009). Use of in vivo phage display to engineer novel adenoviruses for targeted delivery to the cardiac vasculature. FEBS Lett. *583*, 2100-2107.

Ono,Y., Sensui,H., Sakamoto,Y., and Nagatomi,R. (2006). Knockdown of hypoxia-inducible factor-1alpha by siRNA inhibits C2C12 myoblast differentiation. J. Cell Biochem. *98*, 642-649.

Otto,A. and Patel,K. (2010). Signalling and the control of skeletal muscle size. Exp. Cell Res. *316(18),* 3059-66.

Papadimitriou,J.M., Robertson,T.A., Mitchell,C.A., and Grounds,M.D. (1990). The process of new plasmalemma formation in focally injured skeletal muscle fibers. J. Struct. Biol. *103*, 124-134.

Parlakian,A., Gomaa,I., Solly,S., Arandel,L., Mahale,A., Born,G., Marazzi,G., and Sassoon,D. (2010). Skeletal muscle phenotypically converts and selectively inhibits metastatic cells in mice. PLoS. One. *5*, e9299.

Partridge,T.A., Morgan,J.E., Coulton,G.R., Hoffman,E.P., and Kunkel,L.M. (1989). Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature *337*, 176-179.

Pasqualini,R., Moeller,B.J., and Arap,W. (2010). Leveraging molecular heterogeneity of the vascular endothelium for targeted drug delivery and imaging. Semin. Thromb. Hemost. *36*, 343-351.

Pearce,M., Blake,D.J., Tinsley,J.M., Byth,B.C., Campbell,L., Monaco,A.P., and Davies,K.E. (1993). The utrophin and dystrophin genes share similarities in genomic structure. Hum. Mol. Genet. *2*, 1765-1772.

Peault,B., Rudnicki,M., Torrente,Y., Cossu,G., Tremblay,J.P., Partridge,T., Gussoni,E., Kunkel,L.M., and Huard,J. (2007). Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol. Ther. *15*, 867-877.

Perry,R.L. and Rudnick,M.A. (2000). Molecular mechanisms regulating myogenic determination and differentiation. Front Biosci. *5*, D750-D767.

Piitulainen,H., Kivela,R., Komi,P., Kainulainen,H., and Kyrolainen,H. (2008). Molecular adaptations of voltage-gated sodium ion channel related proteins after fatiguing stretch-shortening cycle exercise. Scand. J. Med. Sci. Sports *18*, 636-642.

Pinto,L.C., Favaro,W.J., and Cagnon,V.H. (2010). Proliferative, structural and molecular features of the Mdx mouse prostate. Int. J. Exp. Pathol. *91(5),* 408-19.

Popplewell,L.J., Trollet,C., Dickson,G., and Graham,I.R. (2009). Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. Mol. Ther. *17*, 554-561.

Porter,J.D., Merriam,A.P., Leahy,P., Gong,B., Feuerman,J., Cheng,G., and Khanna,S. (2004). Temporal gene expression profiling of dystrophin-deficient (mdx) mouse diaphragm identifies conserved and muscle group-specific mechanisms in the pathogenesis of muscular dystrophy. Hum. Mol. Genet. *13*, 257-269.

Potthoff,M.J., Wu,H., Arnold,M.A., Shelton,J.M., Backs,J., McAnally,J., Richardson,J.A., Bassel-Duby,R., and Olson,E.N. (2007). Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. J. Clin. Invest *117*, 2459-2467.

Pramono,Z.A., Takeshima,Y., Alimsardjono,H., Ishii,A., Takeda,S., and Matsuo,M. (1996). Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. Biochem. Biophys. Res. Commun. *226(2)*, 445-449.

Qiao,C., Li,J., Jiang,J., Zhu,X., Wang,B., Li,J., and Xiao,X. (2008). Myostatin propeptide gene delivery by adeno-associated virus serotype 8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in mdx mice. Hum. Gene Ther. *19*, 241-254.

Quenneville,S.P., Chapdelaine,P., Skuk,D., Paradis,M., Goulet,M., Rousseau,J., Xiao,X., Garcia,L., and Tremblay,J.P. (2007). Autologous transplantation of muscle precursor cells modified with a lentivirus for muscular dystrophy: human cells and primate models. Mol. Ther. *15*, 431-438.

Quinlan,J.G., Wong,B.L., Niemeier,R.T., McCullough,A.S., Levin,L., and Emanuele,M. (2006). Poloxamer 188 failed to prevent exercise-induced membrane breakdown in mdx skeletal muscle fibers. Neuromuscul. Disord. *16*, 855-864.

Rodino-Klapac,L.R., Montgomery,C.L., Bremer,W.G., Shontz,K.M., Malik,V., Davis,N., Sprinkle,S., Campbell,K.J., Sahenk,Z., Clark,K.R., Walker,C.M., Mendell,J.R., and Chicoine,L.G. (2010). Persistent expression of FLAG-tagged micro dystrophin in nonhuman primates following intramuscular and vascular delivery. Mol. Ther. *18*, 109-117.

Rolland,J.F., De,L.A., Burdi,R., Andreetta,F., Confalonieri,P., and Conte,C.D. (2006). Overactivity of exercise-sensitive cation channels and their impaired modulation by IGF-1 in mdx native muscle fibers: beneficial effect of pentoxifylline. Neurobiol. Dis. *24*, 466-474.

Rybakova,I.N., Humston,J.L., Sonnemann,K.J., and Ervasti,J.M. (2006). Dystrophin and utrophin bind actin through distinct modes of contact. J. Biol. Chem. *281*, 9996-10001.

Sabourin,J., Lamiche,C., Vandebrouck,A., Magaud,C., Rivet,J., Cognard,C., Bourmeyster,N., and Constantin,B. (2009). Regulation of TRPC1 and TRPC4 cation channels requires an alpha1-syntrophin-dependent complex in skeletal mouse myotubes. J. Biol. Chem. *284*, 36248-36261.

Samoylova,T.I. and Smith,B.F. (1999). Elucidation of muscle-binding peptides by phage display screening. Muscle Nerve *22*, 460-466.

Sander,M., Chavoshan,B., Harris,S.A., Iannaccone,S.T., Stull,J.T., Thomas,G.D., and Victor,R.G. (2000). Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. Proc. Natl. Acad. Sci. U. S. A *97*, 13818-13823.

Sandri,M. (2010). Autophagy in health and disease. 3. Involvement of autophagy in muscle atrophy. Am. J. Physiol Cell Physiol *298*, C1291-C1297.

Schultz,E., Jaryszak,D.L., and Valliere,C.R. (1985). Response of satellite cells to focal skeletal muscle injury. Muscle Nerve *8*, 217-222.

Scott,J.K. and Smith,G.P. (1990). Searching for peptide ligands with an epitope library. Science *249*, 386-390.

Seow,Y., Yin,H., and Wood,M.J. (2010). Identification of a novel muscle targeting peptide in mdx mice. Peptides. *31(10),* 1873-7.

Sharp,N.J., Kornegay,J.N., Van Camp,S.D., Herbstreith,M.H., Secore,S.L., Kettle,S., Hung,W.Y., Constantinou,C.D., Dykstra,M.J., Roses,A.D., and . (1992). An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy. Genomics *13*, 115-121.

Shavlakadze,T., Chai,J., Maley,K., Cozens,G., Grounds,G., Winn,N., Rosenthal,N., and Grounds,M.D. (2010). A growth stimulus is needed for IGF-1 to induce skeletal muscle hypertrophy in vivo. J Cell Sci *123*, 960-971.

Shi,S., Hoogaars,W.M., de Gorter,D.J., van Heiningen,S.H., Lin,H.Y., Hong,C.C., Kemaladewi,D.U., Aartsma-Rus,A., Ten,D.P., and 't Hoen,P.A. (2011). BMP antagonists enhance myogenic differentiation and ameliorate the dystrophic phenotype in a DMD mouse model. Neurobiol. Dis. *41(2)*, 353-360.

Shirakabe,K., Wakatsuki,S., Kurisaki,T., and Fujisawa-Sehara,A. (2001). Roles of Meltrin beta /ADAM19 in the processing of neuregulin. J. Biol. Chem. *276*, 9352-9358.

Shkryl,V.M., Martins,A.S., Ullrich,N.D., Nowycky,M.C., Niggli,E., and Shirokova,N. (2009). Reciprocal amplification of ROS and Ca(2+) signals in stressed mdx dystrophic skeletal muscle fibers. Pflugers Arch. *458*, 915-928.

Sirsi,S.R., Schray,R.C., Guan,X., Lykens,N.M., Williams,J.H., Erney,M.L., and Lutz,G.J. (2008). Functionalized PEG-PEI copolymers complexed to exon-skipping oligonucleotides improve dystrophin expression in mdx mice. Hum. Gene Ther. *19*, 795-806.

Skuk,D., Goulet,M., Roy,B., Piette,V., Cote,C.H., Chapdelaine,P., Hogrel,J.Y., Paradis,M., Bouchard,J.P., Sylvain,M., Lachance,J.G., and Tremblay,J.P. (2007a). First test of a "high-density injection" protocol for myogenic cell transplantation throughout large volumes of muscles in a Duchenne muscular dystrophy patient: eighteen months follow-up. Neuromuscul. Disord. *17*, 38-46.

Skuk,D., Paradis,M., Goulet,M., and Tremblay,J.P. (2007b). Ischemic central necrosis in pockets of transplanted myoblasts in nonhuman primates: implications for cell-transplantation strategies. Transplantation *84*, 1307-1315.

Song,W., Kwak,H.B., Kim,J.H., and Lawler,J.M. (2009). Exercise training modulates the nitric oxide synthase profile in skeletal muscle from old rats. J. Gerontol. A Biol. Sci. Med. Sci. *64*, 540-549.

Spallotta,F., Rosati,J., Straino,S., Nanni,S., Grasselli,A., Ambrosino,V., Rotili,D., Valente,S., Farsetti,A., Mai,A., Capogrossi,M.C., Gaetano,C., and Illi,B. (2010). Nitric oxide determines mesodermic differentiation of mouse embryonic stem cells by activating class IIa histone deacetylases: potential therapeutic implications in a mouse model of hindlimb ischemia. Stem Cells *28*, 431-442.

St Pierre,S.J., Chakkalakal,J.V., Kolodziejczyk,S.M., Knudson,J.C., Jasmin,B.J., and Megeney,L.A. (2004). Glucocorticoid treatment alleviates dystrophic myofiber pathology by activation of the calcineurin/NF-AT pathway. FASEB J. *18*, 1937-1939.

Straathof,C.S., Overweg-Plandsoen,W.C., van den Burg,G.J., van der Kooi,A.J., Verschuuren,J.J., and de Groot,I.J. (2009). Prednisone 10 days on/10 days off in patients with Duchenne muscular dystrophy. J. Neurol. *256*, 768-773.

Sun,G., Haginoya,K., Dai,H., Chiba,Y., Uematsu,M., Hino-Fukuyo,N., Onuma,A., Iinuma,K., and Tsuchiya,S. (2009). Intramuscular renin-angiotensin system is activated in human muscular dystrophy. J. Neurol. Sci. *280*, 40-48.

Suzuki,J., Imaizumi,S., Kayama,T., and Yoshimoto,T. (1985). Chemiluminescence in hypoxic brain--the second report: cerebral protective effect of mannitol, vitamin E and glucocorticoid. Stroke *16*, 695-700.

Suzuki,N., Motohashi,N., Uezumi,A., Fukada,S., Yoshimura,T., Itoyama,Y., Aoki,M., Miyagoe-Suzuki,Y., and Takeda,S. (2007). NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. J. Clin. Invest *117*, 2468-2476.

Tanaka,H., Ikeya,K., and Ozawa,E. (1990). Difference in the expression pattern of dystrophin on the surface membrane between the skeletal and cardiac muscles of mdx carrier mice. Histochemistry *93*, 447-452.

Tidball,J.G. and Villalta,S.A. (2010). Regulatory interactions between muscle and the immune system during muscle regeneration. Am. J. Physiol Regul. Integr. Comp Physiol. *298(5),* R1173-87.

Tidball,J.G. and Wehling-Henricks,M. (2007). The role of free radicals in the pathophysiology of muscular dystrophy. J. Appl. Physiol *102*, 1677-1686.

Tinsley,J.M., Potter,A.C., Phelps,S.R., Fisher,R., Trickett,J.I., and Davies,K.E. (1996). Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature *384*, 349-353.

Tinsley,J., Deconinck,N., Fisher,R., Kahn,D., Phelps,S., Gillis,J.M., and Davies,K. (1998). Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nat. Med. *4*, 1441-1444.

Torrente,Y., Belicchi,M., Marchesi,C., Dantona,G., Cogiamanian,F., Pisati,F., Gavina,M., Giordano,R., Tonlorenzi,R., Fagiolari,G., Lamperti,C., Porretti,L., Lopa,R., Sampaolesi,M., Vicentini,L., Grimoldi,N., Tiberio,F., Songa,V., Baratta,P., Prelle,A., Forzenigo,L., Guglieri,M., Pansarasa,O., Rinaldi,C., Mouly,V., Butler-Browne,G.S., Comi,G.P., Biondetti,P., Moggio,M., Gaini,S.M., Stocchetti,N., Priori,A., D'Angelo,M.G., Turconi,A., Bottinelli,R., Cossu,G., Rebulla,P., and Bresolin,N. (2007). Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients. Cell Transplant. *16*, 563-577.

Townsend,D., Yasuda,S., Chamberlain,J., and Metzger,J.M. (2009). Cardiac consequences to skeletal muscle-centric therapeutics for Duchenne muscular dystrophy. Trends Cardiovasc. Med. *19*, 50-55.

Townsend,D., Turner,I., Yasuda,S., Martindale,J., Davis,J., Shillingford,M., Kornegay,J.N., and Metzger,J.M. (2010). Chronic administration of membrane sealant prevents severe cardiac injury and ventricular dilatation in dystrophic dogs. J. Clin. Invest *120*, 1140-1150.

Tuffery,S., Chambert,S., Bareil,C., Sarda,P., Coubes,C., Echenne,B., Demaille,J., and Claustres,M. (1998). Mutation analysis of the dystrophin gene in Southern French DMD or BMD families: from Southern blot to protein truncation test. Hum. Genet. *102(3)*, 334-342.

Turk,R., Sterrenburg,E., de Meijer,E.J., van Ommen,G.J., den Dunnen,J.T., and 't Hoen,P.A. (2005). Muscle regeneration in dystrophin-deficient mdx mice studied by gene expression profiling. BMC. Genomics *6*, 98.

Turner,J.J., Jones,S., Fabani,M.M., Ivanova,G., Arzumanov,A.A., and Gait,M.J. (2007). RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA. Blood Cells Mol. Dis. *38*, 1-7.

Turner,P.R., Westwood,T., Regen,C.M., and Steinhardt,R.A. (1988). Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. Nature *335*, 735-738.

van Deutekom,J.C., Bremmer-Bout,M., Janson,A.A., Ginjaar,I.B., Baas,F., den Dunnen,J.T., and van Ommen,G.J. (2001). Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum. Mol. Genet. *10*, 1547-1554.

van Deutekom,J.C., Janson,A.A., Ginjaar,I.B., Frankhuizen,W.S., Aartsma-Rus,A., Bremmer-Bout,M., den Dunnen,J.T., Koop,K., van der Kooi,A.J., Goemans,N.M., de Kimpe,S.J., Ekhart,P.F., Venneker,E.H., Platenburg,G.J., Verschuuren,J.J., and van Ommen,G.J. (2007). Local dystrophin restoration with antisense oligonucleotide PRO051. N. Engl. J. Med. *357*, 2677-2686.

van Gemert,A.M., van der Laan,A.M., Pilgram,G.S., Fradkin,L.G., Noordermeer,J.N., Tanke,H.J., and Jost,C.R. (2009). In vivo monitoring of mRNA movement in Drosophila body wall muscle cells reveals the presence of myofiber domains. PLoS One *4*, e6663.

van Vliet,V.L., De Winter,C.L., van Deutekom,J.C., van Ommen,G.J., and artsma-Rus,A. (2008). Assessment of the feasibility of exon 45-55 multiexon skipping for Duchenne muscular dystrophy. BMC. Med. Genet. *9*, 105.

Vandebrouck,C., Imbert,N., Duport,G., Cognard,C., and Raymond,G. (1999). The effect of methylprednisolone on intracellular calcium of normal and dystrophic human skeletal muscle cells. Neurosci. Lett. *269*, 110-114.

Vandenburgh,H., Shansky,J., Benesch-Lee,F., Skelly,K., Spinazzola,J.M., Saponjian,Y., and Tseng,B.S. (2009). Automated drug screening with contractile muscle tissue engineered from dystrophic myoblasts. FASEB J. *23*, 3325-3334.

Vidal,B., Serrano,A.L., Tjwa,M., Suelves,M., Ardite,E., De,M.R., Baeza-Raja,B., Martinez de,L.M., Lafuste,P., Ruiz-Bonilla,V., Jardi,M., Gherardi,R., Christov,C., Dierssen,M., Carmeliet,P., Degen,J.L., Dewerchin,M., and Munoz-Canoves,P. (2008). Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway. Genes Dev. *22*, 1747-1752.

Vignaud,A., Hourde,C., Medja,F., Agbulut,O., Butler-Browne,G., and Ferry,A. (2010). Impaired skeletal muscle repair after ischemia-reperfusion injury in mice. J. Biomed. Biotechnol. *2010*, 724914.

Villalta,S.A., Nguyen,H.X., Deng,B., Gotoh,T., and Tidball,J.G. (2009). Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Hum. Mol. Genet. *18*, 482-496.

Wadia,J.S., Stan,R.V., and Dowdy,S.F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat. Med. *10*, 310-315.

Wagner,K.R., Fleckenstein,J.L., Amato,A.A., Barohn,R.J., Bushby,K., Escolar,D.M., Flanigan,K.M., Pestronk,A., Tawil,R., Wolfe,G.I., Eagle,M., Florence,J.M., King,W.M., Pandya,S., Straub,V., Juneau,P., Meyers,K., Csimma,C., Araujo,T., Allen,R., Parsons,S.A., Wozney,J.M., Lavallie,E.R., and Mendell,J.R. (2008). A phase I/IItrial of MYO-029 in adult subjects with muscular dystrophy. Ann. Neurol. *63*, 561-571.

Wakayama,Y., Shibuya,S., Jimi,T., Takeda,A., and Oniki,H. (1993). Size and localization of dystrophin molecule: immunoelectron microscopic and freeze etching studies of muscle plasma membranes of murine skeletal myofibers. Acta Neuropathol. *86*, 567-577.

Wang,B., Li,J., Fu,F.H., and Xiao,X. (2009a). Systemic human minidystrophin gene transfer improves functions and life span of dystrophin and dystrophin/utrophin-deficient mice. J. Orthop. Res. *27*, 421-426.

Wang,B.W., Chang,H., Kuan,P., and Shyu,K.G. (2008a). Angiotensin II activates myostatin expression in cultured rat neonatal cardiomyocytes via p38 MAP kinase and myocyte enhance factor 2 pathway. J. Endocrinol. *197*, 85-93.

Wang,C., Wu,L.L., Liu,J., Zhang,Z.G., Fan,D., and Li,L. (2008b). Crosstalk between angiotensin II and platelet derived growth factor-BB mediated signal pathways in cardiomyocytes. Chin Med. J. (Engl.) *121*, 236-240.

Wang,W., Pan,H., Murray,K., Jefferson,B.S., and Li,Y. (2009b). Matrix metalloproteinase-1 promotes muscle cell migration and differentiation. Am. J. Pathol. *174*, 541-549.

Wang,Q., Yin,H., Camelliti,P., Betts,C., Moulton,H., Lee,H., Saleh,A.F., Gait,M.J., and Wood,M.J. (2010). In vitro evaluation of novel antisense oligonucleotides is predictive of in vivo exon skipping activity for Duchenne muscular dystrophy. J. Gene Med. *12*, 354-364.

Wang,Z., Zhu,T., Qiao,C., Zhou,L., Wang,B., Zhang,J., Chen,C., Li,J., and Xiao,X. (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat. Biotechnol. *23*, 321-328.

Webster,C. and Blau,H.M. (1990). Accelerated age-related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. Somat. Cell Mol. Genet. *16*, 557-565.

Wee,K.B., Pramono,Z.A., Wang,J.L., MacDorman,K.F., Lai,P.S., and Yee,W.C. (2008). Dynamics of co-transcriptional pre-mRNA folding influences the induction of dystrophin exon skipping by antisense oligonucleotides. PLoS. One. *3*, e1844.

Wehling,M., Spencer,M.J., and Tidball,J.G. (2001). A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. J. Cell Biol. *155*, 123-131.

Wehling-Henricks,M., Lee,J.J., and Tidball,J.G. (2004). Prednisolone decreases cellular adhesion molecules required for inflammatory cell infiltration in dystrophin-deficient skeletal muscle. Neuromuscul. Disord. *14*, 483-490.

Wehling-Henricks,M., Jordan,M.C., Gotoh,T., Grody,W.W., Roos,K.P., and Tidball,J.G. (2010). Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in mdx muscular dystrophy. PLoS. One. *5(5)*, e10763.

Weissmann,G. (1976). Corticosteroids and membrane stabilization. Circulation *53*, I171-I172.

Welch,E.M., Barton,E.R., Zhuo,J., Tomizawa,Y., Friesen,W.J., Trifillis,P., Paushkin,S., Patel,M., Trotta,C.R., Hwang,S., Wilde,R.G., Karp,G., Takasugi,J., Chen,G., Jones,S., Ren,H., Moon,Y.C., Corson,D., Turpoff,A.A., Campbell,J.A., Conn,M.M., Khan,A., Almstead,N.G., Hedrick,J., Mollin,A., Risher,N., Weetall,M., Yeh,S., Branstrom,A.A., Colacino,J.M., Babiak,J., Ju,W.D., Hirawat,S., Northcutt,V.J., Miller,L.L., Spatrick,P., He,F., Kawana,M., Feng,H., Jacobson,A., Peltz,S.W., and Sweeney,H.L. (2007). PTC124 targets genetic disorders caused by nonsense mutations. Nature *447*, 87-91.

Welle,S., Burgess,K., Thornton,C.A., and Tawil,R. (2009). Relation between extent of myostatin depletion and muscle growth in mature mice. Am. J. Physiol Endocrinol. Metab. *297(4),* E935-40

Wilton,S.D., Lloyd,F., Carville,K., Fletcher,S., Honeyman,K., Agrawal,S., and Kole,R. (1999). Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides. Neuromuscul. Disord. *9*, 330-338.

Wilton,S.D., Fall,A.M., Harding,P.L., McClorey,G., Coleman,C., and Fletcher,S. (2007). Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. Mol. Ther. *15*, 1288-1296.

Wu,P.I., Minisini,S., and Edelman,E.R. (2009). Intramuscular drug transport under mechanical loading: resonance between tissue function and uptake. J. Control Release *136*, 99-109.

Wu,R.P., Youngblood,D.S., Hassinger,J.N., Lovejoy,C.E., Nelson,M.H., Iversen,P.L., and Moulton,H.M. (2007). Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity. Nucleic Acids Res. *35*, 5182-5191.

Yamada,M., Tatsumi,R., Yamanouchi,K., Hosoyama,T., Shiratsuchi,S., Sato,A., Mizunoya,W., Ikeuchi,Y., Furuse,M., and Allen,R.E. (2010). High concentrations of HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: a possible mechanism for reestablishing satellite cell quiescence in vivo. Am. J. Physiol Cell Physiol *298*, C465-C476.

Yang,Z., Zhu,J., Sriadibhatla,S., Gebhart,C., Alakhov,V., and Kabanov,A. (2005). Promoter- and strain-selective enhancement of gene expression in a mouse skeletal muscle by a polymer excipient Pluronic P85. J Control Release *108*, 496-512.

Yin,H., Moulton,H.M., Seow,Y., Boyd,C., Boutilier,J., Iverson,P., and Wood,M.J. (2008). Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. Hum. Mol. Genet. *17*, 3909-3918.

Yin,H., Moulton,H.M., Betts,C., Seow,Y., Boutilier,J., Iverson,P.L., and Wood,M.J. (2009). A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice. Hum. Mol. Genet. *18(22),* 4405-14.

Yokota,T., Lu,Q.L., Partridge,T., Kobayashi,M., Nakamura,A., Takeda,S., and Hoffman,E. (2009). Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. Ann. Neurol. *65*, 667-676.

Youngblood,D.S., Hatlevig,S.A., Hassinger,J.N., Iversen,P.L., and Moulton,H.M. (2007). Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells. Bioconjug. Chem. *18*, 50-60.

Yu,C.Y., Yuan,Z., Cao,Z., Wang,B., Qiao,C., Li,J., and Xiao,X. (2009). A muscle-targeting peptide displayed on AAV2 improves muscle tropism on systemic delivery. Gene Ther. *16*, 953-962.

Yuasa,K., Hagiwara,Y., Ando,M., Nakamura,A., Takeda,S., and Hijikata,T. (2008). MicroRNA-206 is highly expressed in newly formed muscle fibers: implications regarding potential for muscle regeneration and maturation in muscular dystrophy. Cell Struct. Funct. *33*, 163-169.

Zahid,M., Phillips,B.E., Albers,S.M., Giannoukakis,N., Watkins,S.C., and Robbins,P.D. (2010). Identification of a cardiac specific protein transduction domain by in vivo biopanning using a m13 phage Peptide display library in mice. PLoS. One. *5*.

Zhang,L., Hoffman,J.A., and Ruoslahti,E. (2005). Molecular Profiling of Heart Endothelial Cells. Circulation. *112(11),* 1601-11.

Zhao,X., Pan,F., Holt,C.M., Lewis,A.L., and Lu,J.R. (2009). Controlled delivery of antisense oligonucleotides: a brief review of current strategies. Expert. Opin. Drug Deliv. *6*, 673-686.

Zhou,L., Rafael-Fortney,J.A., Huang,P., Zhao,X.S., Cheng,G., Zhou,X., Kaminski,H.J., Liu,L., and Ransohoff,R.M. (2008). Haploinsufficiency of utrophin gene worsens skeletal muscle inflammation and fibrosis in mdx mice. J. Neurol. Sci. *264*, 106-111.

Zohar,R., Zhu,B., Liu,P., Sodek,J., and McCulloch,C.A. (2004). Increased cell death in osteopontin-deficient cardiac fibroblasts occurs by a caspase-3-independent pathway. Am. J. Physiol Heart Circ. Physiol *287*, H1730-H1739.

Zou,Y., Akazawa,H., Qin,Y., Sano,M., Takano,H., Minamino,T., Makita,N., Iwanaga,K., Zhu,W., Kudoh,S., Toko,H., Tamura,K., Kihara,M., Nagai,T., Fukamizu,A., Umemura,S., Iiri,T., Fujita,T., and Komuro,I. (2004). Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. Nat. Cell Biol. *6*, 499-506.