CHAPTER 1

TGF-β SIGNALING IN DUCHENNE MUSCULAR DYSTROPHY

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Signal transduction pathways and the (human) body resemble electrical circuit in complex machinery. The variety of signal transduction processes is required for coordinating individual cells to support the organism as a whole. Many diseases arise from defects or improper activation of such pathways, highlighting the importance of this process in biology and medicine.

The Transforming Growth Factor-beta (TGF-β) pathway is one example of how cells communicate. It involves secretion of extracellular signaling molecules or ligands in the latent form, which are subsequently activated and binding to their cell-surface receptors to trigger events inside the cell. This chapter will elaborate on the TGF-β signaling mechanism and how it regulates myriad of cellular responses, with focus on normal skeletal muscle regeneration process and disease such as Duchenne muscular dystrophy (DMD). Several examples of translating knowledge in the TGF-β signal transduction into therapy for DMD will also be given towards the end of this chapter. In addition, an overview of potential therapeutic approaches that are aimed to tackle the genetic cause of DMD will be presented.

1.1 TGF-β signaling

The TGF-β superfamily consists of 33 secreted cytokines in human, which include the TGF-β1, -2, -3, Bone Morphogenetic Proteins (BMPs), Growth and Differentiation Factors (GDFs), Activins/inhibins and nodal-related proteins (Feng and Derynck, 2005). Most of these members are synthesized as latent proteins, which are proteolytically cleaved for activation and form functional dimers that initiate downstream signaling through specific receptor interactions. These dimeric ligands bind and assemble hetero-tetrameric complexes of two types of transmembrane serine/threonine kinase receptors, designated as the type I and type II receptors (**Figure 1.1**).

Five type II receptors, namely Activin receptor IIA and IIB (ACVR2A, ACVR2B), TGF-β receptor II (TGFBR2), BMP receptor II (BMPR2), Müllerian inhibiting substance receptor II (MISR2) and seven type I receptors, which are termed activin-receptor like kinases (ALK 1-7, see **Table 1** for official gene symbols) have been identified with different combinations of type I/II receptors mediating the activity of different family member proteins.

Activin initiates signaling by binding to ACVR2A/B, in combination with type 1 receptors ALK4 and ALK7. TGF-β itself mainly uses TGFBR2 and ALK5, with an exception in endothelial cells in which it can signal via ALK1 (Goumans *et al.*, 2003; Lebrin *et al.*, 2004). Similar to activin, myostatin (also known as GDF-8), can bind to ACVR2A, ACVR2B and ALK4, but is also able to signal via ALK5 (Rebbapragada *et al.*, 2003). BMP ligands form complexes with other receptor combinations, namely the type II receptors BMPR2 or ACVR2A/B with type I receptors ALK1, ALK2, ALK3 and ALK6 (Feng *et al.*, 2005). Ligand-receptor complex formation leads to activation of the type I receptor through type II receptor-mediated phosphorylation of serine and threonine residues in the GS domain. The activated type I receptor will subsequently phosphorylate the intracellular receptor-regulated Smad (R-Smad) proteins. Upon association of R-Smads with the common Smad protein (Smad4), the heteromeric Smad complexes translocate into the nucleus, where they regulate the transcription of a multitude of target genes. Different R-Smads are activated by different ligands, with activin, TGF-β and myostatin signaling being mediated via Smad2 and Smad3, and BMP signaling via Smad 1/5/8 (Shi and Massague, 2003).

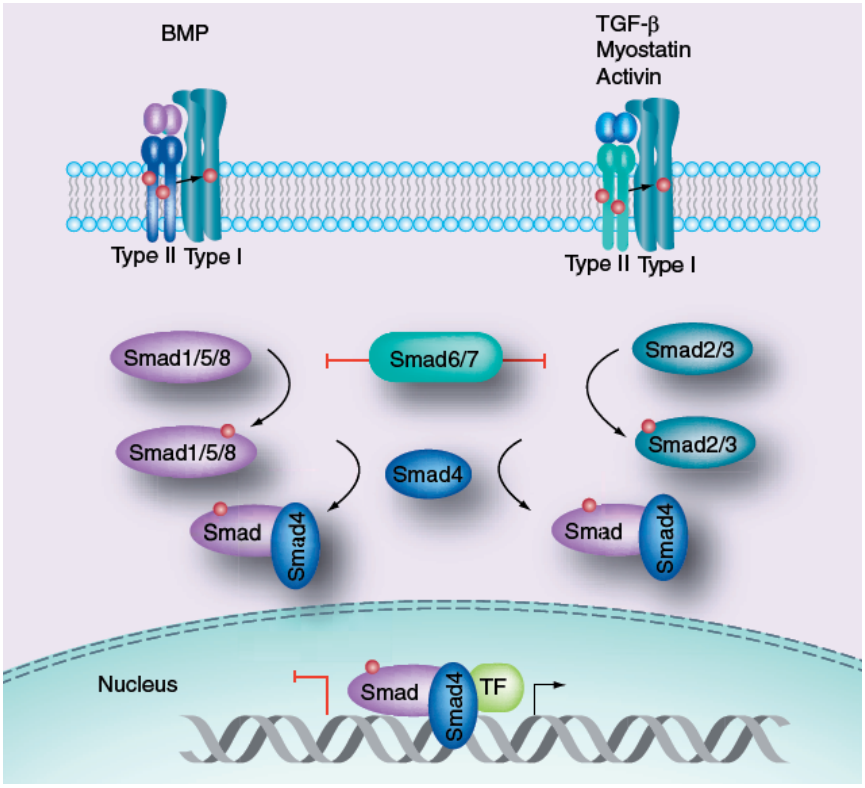

Figure 1.1. Cartoon of the TGF-ß-Smad signaling pathway. TGF-β family proteins bind to transmembrane serine/threonine kinase receptors. The type II receptor phosphorylates (red circles indicate the phosphorylation) the type I receptors and induces activation of regulatory Smad proteins (Smad 1/5/8 or Smad 2/3) by phosphorylation. This can be inhibited by Smad6/7, the inhibitory Smad. The common Smad (Smad 4) binds and forms a complex prior to translocation into the nucleus. The Smad complex binds to transcription factor (TF) and regulates the expression of the target genes. BMP-proteins use distinct receptors and activate different Smad complexes than TGF-β/Myostatin/Activin.

Table 1. TGF-β family members, type II, type I and interacting proteins.

Ligands	Type II	Type I (official name)	Co-receptors / Interacting proteins (OFficial Name)
TGF-β 1,2,3	TGFBR2	ALK5 (TGFBR1), ALK1 (ACVRL1)	Endoglin (ENG), Betaglycan (TGFBR3), Cripto (TDGF1), Decorin (DCN)
Myostatin	ACVR2A ACVR2B	ALK4 (ACVR1B), ALK5	Cripto, Decorin, Follistatin (FST)
Activin	ACVR2A ACVR2B	ALK4, ALK7 (ACVR1C)	Cripto, Follistatin
BMP	BMPR2 ACVR2A, ACVR2B	ALK1, ALK2 (ACVR1A), ALK3 (BMPR1A), ALK6 (BMPR1B)	RGMA (RGMA), Dragon (RGMB), RGMC (HFE2), BAMBI (BAMBI), Chordin (CHRD), Noggin (NOGGIN), Follistatin

In addition to the combination of type I/II receptors, the TGF-β signaling pathways are further fine-tuned at multiple levels, such as by presence of extracellular antagonists that modify ligand activity and availability (Moustakas and Heldin, 2009).

Furthermore, several co-receptors can either increase or decrease the affinity of ligands for the type I/II receptor binding. Tissue specificity of the TGF-β family is also achieved by spatio-temporal expression of these modulatory proteins. An overview of TGF-β family members, their receptors, co-receptors and interacting proteins is presented in **Table 1**.

1.2 Role of TGF-β signaling in adult skeletal muscle regeneration and regulation of muscle mass

Postnatal skeletal muscle growth and maturation occurs due to the activity of the muscle progenitor cells, called satellite cells, which have the ability to proliferate upon activation, differentiate, fuse and form new muscle fibers. Adult muscle growth is mainly attributed to muscle fiber hypertrophy, *i.e.* increased muscle mass, and a recent study showed that this process does not require the activity of satellite cells (McCarthy *et al.*, 2011). However, during muscle damage activated satellite cells, or myoblasts, are indispensable for muscle regeneration and are involved in the repair of damaged muscle fibers and the formation of new fibers. Several other muscle resident cells, including endothelial cells, fibroblasts, macrophages and lymphocytes contribute to this process in a complex interplay with muscle satellite cells. Several members of the TGF-β family are known to regulate muscle regeneration/differentiation and muscle mass and are involved in muscle pathologies. To understand the role of TGF-β family members in muscle pathology it is crucial to understand their function in healthy and regenerating muscle, which will be discussed next.

1.2.1 TGF-β

TGF-β has long been described to negatively regulate differentiation of myogenic precursor cells or myoblasts by repressing the transcription and activity of myogenic transcription factors such as *MyoD* and *Myog* (Olson *et al.*, 1986; Massague *et al.*, 1986). In adult muscle, the expression of *TGF-β1, TGF-β2* and *TGF-β3* is induced in damaged and regenerating skeletal muscle and expression of *TGF-β1* is correlated with connective tissue fibrosis (McLennan and Koishi, 1997; Li *et al.*, 2004). Importantly, TGF-β1 can induce fibrosis by stimulating fibroblast proliferation and transdifferentiation of myoblast into myofibroblasts, which results in excessive deposition of extracellular matrix proteins (Li and Huard, 2002; Cencetti *et al.*, 2010). During aging higher levels of TGF-β are correlated with age-related muscle loss, or sarcopenia, and inhibition of TGF-β was found to improve muscle regeneration in old mice (Beggs *et al.*, 2004; Carlson *et al.*, 2008; Carlson *et al.*, 2009). These studies suggest that TGF-β potentially has a pathological role during adult muscle repair and aging by repressing myogenic differentiation and proliferation of satellite cells and stimulating fibrosis. However, several studies also suggest a physiological role for TGF-β signaling in skeletal muscle. Interestingly, other studies showed that low doses of TGF-β1 did not inhibit, but actually stimulate primary myoblast proliferation (Carlson *et al.*, 2009). In addition, the effect of TGF-β on myogenic differentiation depends on cell-to-cell contact, as well as the presence and absence of growth factors (Zentella and Massague, 1992; De *et al.*, 1998). Moreover, TGF-β1 expression in developing muscle is correlated with the development of fast-type muscle (McLennan *et al.*, 1997). Inhibition of TGF-β1 during muscle

regeneration induced the expression of slow-type myosin heavy chain (MHC), suggesting a role in the fiber-type identity (Noirez *et al.*, 2006).

Involvement of downstream targets of TGF-β signaling in muscle regeneration has also been described. In particular, a recent study described TGF-β-activated kinase 1 (TAK1) to be upregulated in skeletal muscle of adult mice during muscle regeneration. Upon genetic ablation of TAK1, MyoD-driven fibroblast to myoblast conversion was also abrogated, suggesting the involvement of TAK1 as regulator of skeletal muscle regeneration and differentiation (Bhatnagar *et al.*, 2010).

1.2.2 Myostatin

Myostatin, as the name indicated, is a negative regulator of adult skeletal muscle mass. Mutations that result in non functional myostatin lead to a hypermuscular phenotype in mice, cattle, sheep, dogs and human (McPherron *et al.*, 1997; Grobet *et al.*, 1997; Kambadur *et al.*, 1997; Schuelke *et al.*, 2004; Clop *et al.*, 2006; Mosher *et al.*, 2007). Muscles of mice with a homozygous deletion in the myostatin gene weigh approximately 2-3 times more than the wild-type littermates (McPherron *et al.*, 1997), which is a result of both muscle hyperplasia and hypertrophy. The role of myostatin in satellite cells was first studied on isolated muscle fibers from myostatin knockout mice. An increase was observed in the number of satellite cells adherent to these fibers, in addition to a higher number of activated satellite cells compared with the wild-type mice (McCroskery *et al.*, 2003). Moreover, myostatin-deficient satellite cells proliferate and differentiate more rapidly than those from wild-type mice, and myostatin was found to inhibit satellite cell proliferation (McCroskery *et al.*, 2003). Furthermore, myostatin suppressed self-renewal of the satellite cell pool by inhibiting Pax7 expression (McFarlane *et al.*, 2008). Therefore, it was proposed that myostatin negatively regulates satellite cell activation by inhibition of cell cycle progression and inhibits satellite cell self-renewal. However, the effect of myostatin on satellite cells is controversial, since another more recent study showed that knockout of myostatin does not result in a difference of satellite cell number and activation in mice (Amthor *et al.*, 2009). Also myostatin did not inhibit myoblast proliferation of primary adult myoblasts in this study, suggesting that these cells are not responsive to myostatin (Amthor *et al.*, 2009). Correspondingly, expression of the main type II receptors necessary for myostatin signaling, *ACVR2A* and *ACVR2B*, was nearly undetectable in adult myoblasts, even though these cells were previously reported to express myostatin (McCroskery *et al.*, 2003; Amthor *et al.*, 2009).

Similarly, the effects of myostatin on satellite cell activity *in vivo* remains controversial. Muscle regeneration was found to be enhanced in myostatin knockout mice and this effect was sustained in old mice, suggesting that myostatin is indeed involved in controlling satellite cell activity (McCroskery *et al.*, 2005; Wagner *et al.*, 2005; Zhu *et al.*, 2007; Amthor *et al.*, 2009). However, others found that knockout of myostatin did not enhance muscle regeneration in dystrophic *mdx* mice and that satellite cells are dispensable for the muscle hypertrophy induced by the absence of myostatin in wild-type mice (Amthor *et al.*, 2009). Interestingly, myostatin may also act as a pro-fibrotic factor and in addition regulate satellite cell and macrophage migration during muscle regeneration. Gastrocnemius muscle of myostatin knockout mice developed significantly less fibrosis and regenerated better compared to wild-type mice, suggesting the importance of myostatin in fibrotic response in regenerating muscle (McCroskery *et al.*, 2005; Zhu *et al.*, 2007). Moreover, myostatin and TGF-β1 are co-localized in degenerative myofibers during muscle regeneration and *in vitro* experiments in myoblasts suggested that myostatin

and TGF-β1 can regulate each other's expression (Zhu *et al.*, 2007). We recently identified some important determinants that distinguish myostatin signaling in myoblasts from non-myogenic cells. We found that ALK4 is the main type I receptor utilized by myostatin in myoblasts, whereas ALK5 is preferred in non myogenic cells, such as fibroblasts (Kemaladewi *et al.*, 2011a). This mechanism was found to be controlled by the co-receptor Cripto that is expressed in adult myoblasts and but not in non myogenic cells, including muscle fibroblasts. We showed that Cripto is required for myostatin signaling in myoblasts and enhances myostatin activity, which distinguishes the signaling mechanism of myostatin from other TGF-β ligands (Kemaladewi *et al.*, 2011a). The potential role of Cripto in muscle regeneration and regulation of muscle mass as well as the spatiotemporal expression of Cripto *in vivo* in skeletal muscle is as yet unclear. However, the observed presence of Cripto in satellite cells and a subset of regenerating muscle fibers (our unpublished observation) suggest a role for this co-receptor in muscle regeneration.

In addition to the regulation of muscle mass and regeneration, myostatin has also been suggested to regulate fiber type specification, as observed in myostatin-deficient mice and cattle, in which an increase of fast glycolytic type IIB fibers and a decrease of type IIA and type I fibers were observed (Martyn *et al.*, 2004; Girgenrath *et al.*, 2005; Hennebry *et al.*, 2009). However, postnatal inhibition of myostatin resulted in a comparable hypertrophy of both fiber types (Girgenrath *et al.*, 2005; Cadena *et al.*, 2010), suggesting that myostatin exerts its effect on fiber type specification during development.

The intracellular effectors of TGF-β/myostatin, *i.e.* Smad2 and 3, have also been implicated in controlling adult muscle mass. Strikingly, Smad3-null mice did not show enhanced muscle mass, but instead showed reduced muscle mass or muscle atrophy, which was correlated with an increase in myostatin expression (Ge *et al.*, 2011). In another study, Smad2/3 inhibition was found to promote muscle hypertrophy. This was found to be independent of satellite cells activity but partially dependent of Akt/mTOR signaling (Sartori *et al.*, 2009), suggesting a cross-talk mechanism between the canonical Smad-dependent pathway and non Smad pathways.

1.2.3 Other ligands

In addition to TGF-β and myostatin, other TGF-β family members have been suggested to regulate muscle mass. Transgenic mice over-expressing ligand binding protein follistatin, which is known to antagonize multiple TGF-β family members, resulted in excessive muscle growth beyond the effect of myostatin knockout (Lee, 2007). Furthermore, transgenic overexpression of follistatin in mice accelerates muscle regeneration (Zhu *et al.*, 2011). Conversely, follistatin heterozygote mice show reduced muscle mass, impaired muscle regeneration and interestingly also a shift towards oxidative fiber types (Lee *et al.*, 2010). In addition to being antagonist of BMP (Amthor *et al.*, 2002) and myostatin (Amthor *et al.*, 2004), follistatin can directly bind to activin (Hashimoto *et al.*, 2000) and mask the type I/II binding sites, thereby abolishing the interaction with activin receptors (Harrington *et al.*, 2006). As the same type I/II receptors are utilized by both myostatin and activin, most likely the same antagonizing mechanisms apply for both ligands. *In vitro,* follistatin is able to block inhibitory activity of myostatin, activin and also TGF-β1 on myoblast differentiation (Zhu *et al.*, 2011). In accordance with the effect of follistatin, myostatin knockout mice treated with the soluble activin type II receptor showed additional muscle growth compared with non-treated mutant mice (Lee *et al.*, 2005). Lee *et al* also generated several mouse models carrying targeted deletions of Inhibin-β subunits, the constituents of activin A, and showed that mice heterozygous for the Inhibin-βA mutation had

increased muscle mass ranging from approximately 8-11% depending on the muscle type (Lee *et al.*, 2010). Mutations in the other subunits had little or no effect. Together these studies suggest that both myostatin and activin are involved in regulation of muscle mass.

In addition to activin, GDF11 was suggested to be a key player in muscle differentiation (Souza *et al.*, 2008). However, direct comparison of genetically ablated myostatin and GDF11 mice showed that both ligands have redundant functions in skeletal patterning but not in muscle size, fiber number and type (McPherron *et al.*, 2009).

BMPs are known to provide spatiotemporal control of myogenic differentiation during development (Amthor *et al.*, 1998), but their function in adult muscle remains relatively obscure. A regulatory role for BMP signaling in satellite cells has been described recently. BMP signaling sustains satellite cells division and inhibits differentiation (Ono *et al.*, 2011; Friedrichs *et al.*, 2011). The mechanism by which BMP inhibits terminal differentiation might be achieved via upregulation of Chordin, BMP modulatory proteins/inhibitors, thereby providing a negative feedback mechanism (Friedrichs *et al.*, 2011). Alternatively, it has been described that Notch signaling is required for BMP4-mediated inhibition of differentiation, providing an extra control mechanism via crosstalk between these two signaling pathways (Dahlqvist *et al.*, 2003). Inhibition of BMP signaling during muscle regeneration resulted in increased fibrosis and reduced fiber size, suggesting an important role of BMP signaling during muscle regeneration (Ono *et al.*, 2011). However, the precise role of individual BMP-ligands and downstream BMP signaling during muscle regeneration and regulation of muscle mass remains to be elucidated.

In summary, TGF-β family members and the components of the signaling pathway play important roles in adult skeletal muscle growth and regeneration. It is therefore not surprising that the expression levels of TGF-β signaling also plays role in the pathophysiology of muscle disorders where the muscle regeneration process is impaired, such as in Duchenne muscular dystrophy.

1.3 Duchenne muscular dystrophy (DMD)

DMD is an X-linked recessive neuromuscular disorder with an incidence of 1:3500 newborn boys. It is caused by mutations in the *DMD* gene that encodes for dystrophin, an important muscle structural protein (**Figure 1.2**). The lack of dystrophin underlies the muscle weakness and rapidly progressing muscle wasting, which are the prominent characteristics of the disease. The patients lose their ambulation around the age of 10 and eventually develop respiratory failure and cardiomyopathy (Emery A.E., 1993). A milder variant of the disease, Becker muscular dystrophy (BMD) arises from similar mutations in the *DMD* gene, but retain the open reading frame, resulting in shorter but partially functional dystrophin protein.

1.3.1 DMD pathology

The pathology of DMD involves continuous muscle de-/regeneration process, ongoing inflammatory response and abnormal accumulation of connective tissue or fibrosis (**Figure 1.3**), which are heavily intertwined. Without functional dystrophin, dystrophic muscle membranes are leaky, resulting in muscle fiber damage and degradation. This calls for excessive and continual demand of repair by satellite cells, leading to their deficiency and exhaustion, causing a reduction in the regenerative capacity of the muscle and an imbalance between degradation and repair (Blau *et al.*, 1983; Sacco *et al.*, 2010). It has been long described that DMD-derived

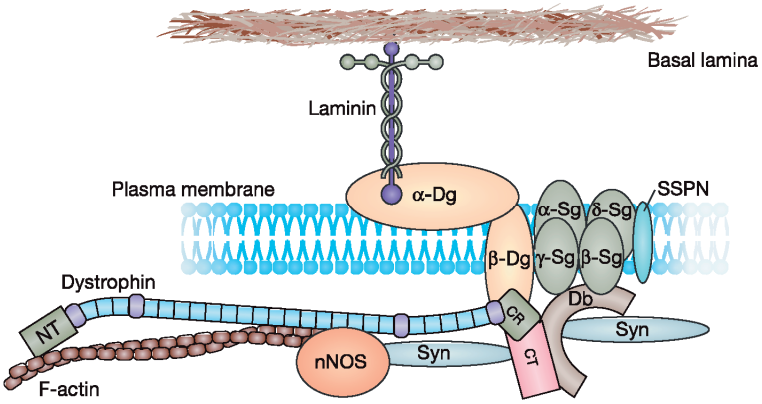

Figure 1.2. Schematic drawing of Dystrophin-associated Glycoprotein Complex (DGC). DGC consists of various proteins that interact with each other, in which alteration of the constituents leads to different type of muscular dystrophies. Dystrophin connects cystokeletal protein F-actin to proteins at the sarcolemma of muscle membrane, including β-dystroglycan (β-Dg) and, via dystrobrevin (Db), to sarcoglycans (γ-Sg, β-Sg). The N-, C-terminus (NT, CT) and cysteine-rich (CR) domains of dystrophin are essential for this function, whereas the central rod composed of 24 spectrin-like domains and 4 hinge domains are to some extent redundant. Other abbreviations: nNOS: neuronal nitric oxide synthase; Syn: syntrophin; SSPN: sarcospan. Figure was adapted from (Muir and Chamberlain, 2009).

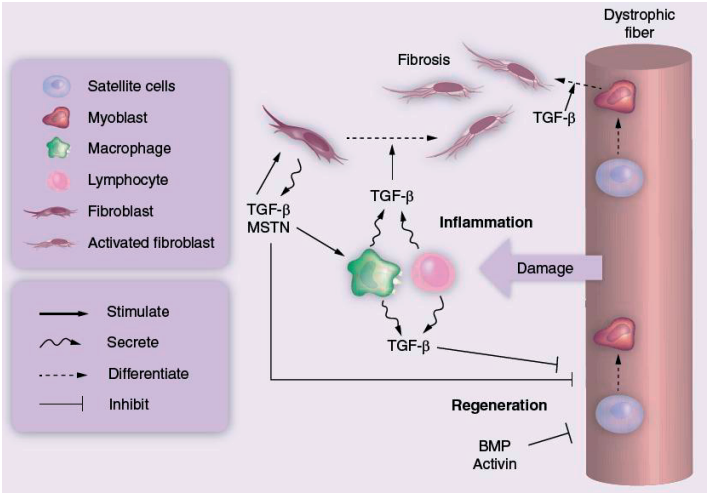

Figure 1.3. Influence of TGF-ß family members in DMD pathology. Muscle fibers lacking dystrophin are leaky and more prone to contraction-induced damage, which leads to activation of surrounding immune cells, such as macrophages and lymphocytes. Pro-fibrotic cytokines secreted by these immune cells, such as TGF-β induce proliferation and activation of fibroblasts, inhibit satellite cells activation and myoblast fusion, as well as inducing satellite cells to fibroblast transdifferentiation. Myostatin is secreted by and directly regulates fibroblasts proliferation and activation. Myostatin can also induce the secretion of proinflammatory cytokines and macrophages migration. Finally, BMP and activin also have inhibitory roles in muscle growth, possibly by repressing satellite cells activation and myoblast fusion.

satellite cells showed decline in their replicative life-span (Blau *et al.*, 1983; Webster and Blau, 1990). However, there are some discrepancies in the literature. Some studies suggested that the number of satellite cells per fiber does not correlate with the proliferation defect. In fact, there is a significant increase in the number of satellite cells in the dystrophic muscle fiber population (Watkins and Cullen, 1988; Kottlors and Kirschner, 2010). However, other studies showed that dystrophic muscles have ~25% fewer nuclei per myotube compared with controls, with higher cellular heterogeneity between fibroblasts and nonfusing myoblasts (Delaporte *et al.*, 1984). A currently accepted explanation of satellite cell exhaustion in DMD is due to telomere length shortening, possibly as a result of increased turnover. It has been reported in aged *mdx* mice (Lund *et al.*, 2007), a mouse model for DMD (see below) and to a certain extent in DMD patient muscle biopsies (Oexle *et al.*, 1997; Aguennouz *et al.*, 2010). Importantly, mice have longer telomeres compared to humans, which may account for the much milder dystrophic phenotype of *mdx* mice (Sacco *et al.*, 2010). In concordance to this notion, *mdx* mice lacking the RNA component of telomeres (*mdx/mTR* mice) show a more severe dystrophic phenotype that progressively worsens with age (Sacco *et al.*, 2010). Myoblasts isolated from these mice have shortened telomeres and show proliferation defects *in vitro* as well as decreased myogenic potential *in vivo* (Sacco *et al.*, 2010).

Subsequent to tissue damage, muscle-resident immune cells will be activated in DMD muscle and migrate chemotactically towards the site of injury. The nature, duration and intensity of the inflammatory response after muscle damage and regeneration can crucially influence the outcome of muscle repair or fibrosis (Tidball and Villalta, 2010). Macrophages and lymphocytes seem to be the dominant immune cells to stimulate these processes. Cytokines secreted by macrophages play important roles in satellite cells activation and myogenesis both *in vitro* and *in vivo* (Cantini and Carraro, 1995; Massimino *et al.*, 1997; Cantini *et al.*, 2002). Furthermore, macrophages secrete TGF-β, matrix metalloproteases (MMPs) and Tissue Inhibitors of Metalloproteases (TIMPs), which are all involved in critical process in fibrosis, such as stimulation of fibroblast proliferation and matrix remodeling (Wynn, 2008; Tidball *et al.*, 2010).

One apparent characteristic of dystrophic muscle is the replacement of muscle fiber by fibrotic tissue. This fibrotic process itself is inseparable from muscle degeneration and inflammation described above. Activated fibroblasts are the major cellular source of different types of collagens, fibronectin, proteoglycans and laminin, which together compose the extracellular matrix (Kalluri and Neilson, 2003). A recent report shows that fibroblasts derived from DMD patients are less susceptible to cell death, more adhesive and able to migrate faster than control fibroblasts, which altogether may contribute to muscle fibrosis (Zanotti *et al.*, 2011). In addition, resident fibroblasts are not the only cell type that contributes to fibrosis during muscle regeneration. Local mesenchymal cells, such as myoblasts, can undergo a fibrotic-phenotypic switch upon extended contact with pro-fibrotic cytokines and may, therefore, also contribute to fibrosis in DMD (Li *et al.*, 2002; Li *et al.*, 2004).

In addition to the aforementioned processes, excess calcium enters damaged muscle fibers, likely due to hyperactivity of stretch activated calcium channels. This activates calpains and leads to mitochondrial damages and oxidative stress, further exacerbating fibrosis (Alderton and Steinhardt, 2000; Chen *et al.*, 2000).

DMD animal models provide important knowledge on different aspects of the disease pathology. The most commonly used mouse in DMD research is the *mdx* mouse, which harbors

a point mutation in exon 23 of the *Dmd* gene, leading to formation of a premature stop codon and preventing dystrophin synthesis (Bulfield *et al.*, 1984). From the age of 4 weeks onwards, there are repeated cycles of de-/regeneration and fibrotic tissue depositions become apparent at the later age, in particular in the diaphragm muscle. However, the pathology is less severe compared to the DMD patients and the lifespan is comparable to the wild-type mice. This discrepancy is at least partly caused by longer telomeres as mentioned above, but also by the upregulation of dystrophin homolog utrophin that can partly compensate for the loss of dystrophin in mice. *Mdx* mice with genetically ablated utrophin (*mdx/utrn*$^{-/-}$) have decreased life expectancies and more severe phenotypes (Deconinck *et al.*, 1997; Grady *et al.*, 1997).

1.3.2 Involvement of TGF-β family members in DMD pathology

Upregulation of TGF-β family signaling components are observed in DMD. This also correlates with the disease progression in DMD animal models, in which higher expression levels of *TGF-β1* and TGF-β type I and II receptors (*ALK5* and *Tgfbr2*) is correlated with the severity of the dystrophic phenotype in *mdx* and *mdx/utrn*$^{-/-}$ (**Figure 1.4** and (Zhou *et al.*, 2006)).

TGF-β1 levels were found to be increased in the conditioned medium of DMD-derived myoblasts compared to that of healthy individuals. This would partially explain the reduced fusion and differentiation of DMD-derived satellite cells (Melone *et al.*, 1999). In concordance, *TGF-β1* expression levels in skeletal muscle biopsies of DMD patients were correlated with the degree of fibrosis (Bernasconi *et al.*, 1995), further supporting evidence of a pathogenic role of this cytokine. Extensive mRNA profiling using muscles of different stage of DMD showed that TGF-β pathway was strongly induced in symptomatic patients, but myostatin was not induced during any stage of the disease (Chen *et al.*, 2005). This is in agreement with our observation that *myostatin* expression and the expression of myostatin receptors were not induced in *mdx* or *mdx/utrn*$^{-/-}$ muscle, suggesting no direct correlation with the dystrophic pathology (**Figure 1.4**). Nonetheless, myostatin knockout in *mdx* mice was reported to result in improvement of muscle histology and muscle function (Wagner *et al.*, 2002).

Immune cells, such as macrophages, T- and B-cell lymphocytes secrete TGF-β and express TGF-β receptors. Upon stimulation with fibrinogen, *mdx*-derived macrophages secrete more TGF-β and further enhance collagen production by the resident fibroblast, which is counteracted by fibrinogen loss-of-function in *mdx* mice (Vidal *et al.*, 2008). Depletion of circulating macrophages by inducing nitric oxide in *mdx* mice from age 1 to 4 weeks significantly suppressed muscle necrosis and degeneration, suggesting that macrophages may contribute to muscle damage at the early stage of the disease (Wehling *et al.*, 2001). Immuno-deficient *mdx* mice lacking functional T- and B-lymphocytes were generated and characterized to determine the role of lymphocytes in DMD (Farini *et al.*, 2007). Compared to their *mdx* counterparts, these mice exhibit less diaphragm fibrosis at 12 months and decreased levels of TGF-β1 protein in the muscles. In addition, a subset of T-cells expressing high levels of osteopontin, which plays a role in immune cell migration and survival, was found in *mdx* muscle (Vetrone *et al.*, 2009). Ablation of osteopontin in *mdx* mice resulted in reduction of TGF-β signaling and fibrosis (Vetrone *et al.*, 2009). The pro-fibrotic role of TGF-β1 was also determined in primary DMD fibroblasts and myoblasts.

Interestingly, the proliferation rate and the expression of extracellular matrix components and *myostatin* are significantly increased in muscle- derived DMD fibroblasts compared with control fibroblasts. They were also more sensitive to TGF-β1 treatment (Zanotti *et al.*, 2010).

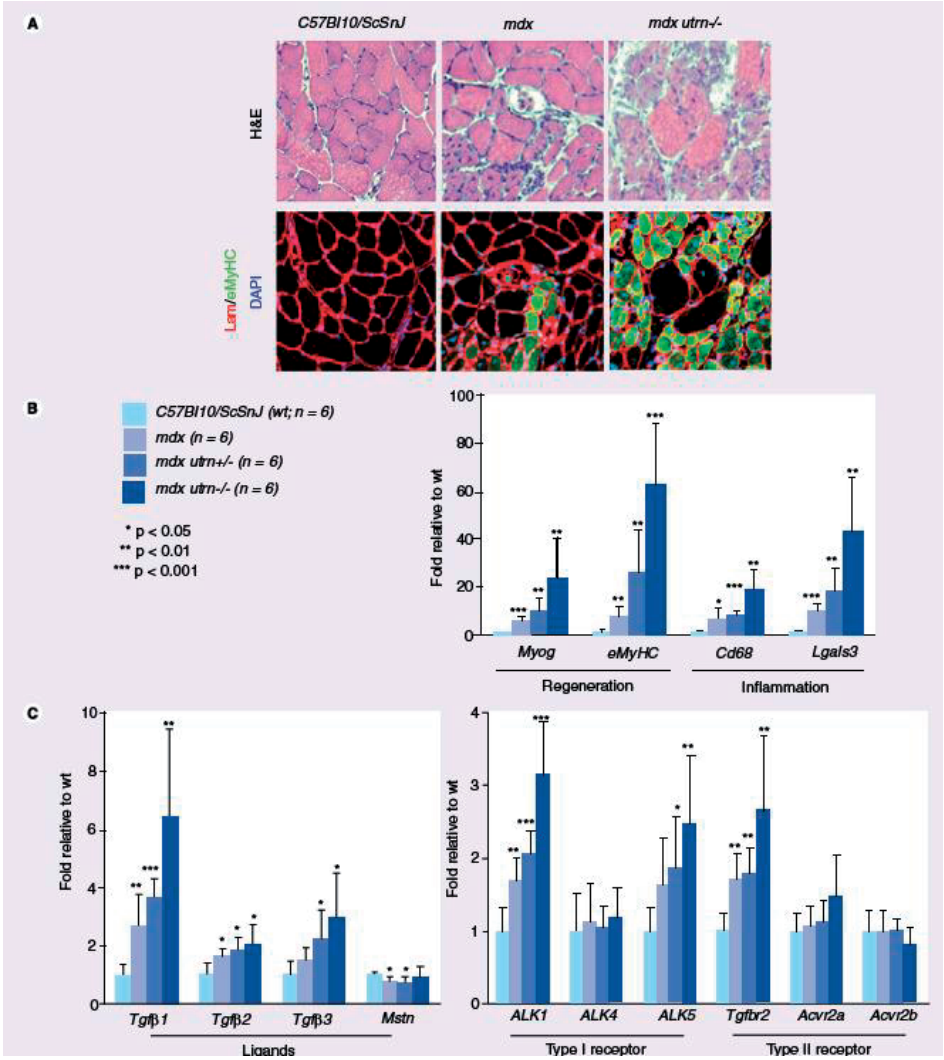

Figure 1.4. TGF-ß family signaling components in DMD mouse models. Comparison of gastrocnemius muscle from 6 weeks-old wild-type (*C57Bl10/ScSnJ*) and DMD mouse models: *mdx*, *mdx utrn +/-* and the most severe *mdx utrn -/-*. Fibrotic areas and regeneration increase as demonstrated by H&E and embryonic myosin heavy chain staining (eMyHC, green) stainings, respectively (Lam=Laminin, red) (A). Gene expression analysis showed that regeneration markers *Myog* and *eMyHc*, as well as inflammation markers *CD68* and *Lgals3* increase in a dose-dependent manner (B). This is correlated with increased expression of all three isoforms of TGF-β, their type I (ALK1 and ALK5) and type II receptors (Tgfbr2). The expression levels of myostatin, its type II receptor (Acvr2a, Acvr2b) and one of the type I receptors ALK4 expression were not altered (C).

Moreover, DMD myotubes also showed increased expression of both *myostatin* and *TGF-β1* and extracellular matrix components such as collagens, MMP-2 and TIMP 1/2 (Zanotti *et al.*, 2007). In addition, myostatin may also play role in the immune response in dystrophic muscle by regulating cytokine expression. In cultured muscle cells, myostatin expression is increased by stimulation with TNF-α via an NF-ΚB-dependent pathway and, exogenous myostatin stimulation enhances IL-6 production via p38 MAPK and MEK1 pathways (Zhang *et al.*, 2011). Furthermore, the number of inflammatory cells decreased more rapidly in muscles of myostatin mutant mice (McCroskery *et al.*, 2005) and antagonism of myostatin in aging mouse muscle leads to enhanced macrophage cell migration *in vivo* (Siriett *et al.*, 2007). It also directly induces fibroblast growth and stimulates skeletal muscle fibrosis (Li *et al.*, 2008). In line with this finding, several studies show that *mdx* mice receiving myostatin inhibitors show suppression of muscle fibrosis, suggesting a role for myostatin in fibrotic progression in DMD, which is discussed in more detail in the next section.

Other ligands of the TGF-β family have been identified as potential players in DMD pathology by gene expression profiling. Expression of BMP ligands, such as *BMP4* and *BMP15* were found to be elevated in DMD-derived myoblasts and *mdx* muscles, respectively (Turk *et al.*, 2005; Sterrenburg *et al.*, 2006; Pescatori *et al.*, 2007). Similarly, expression profiling in DMD patients muscle biopsies also revealed altered BMP signaling, with higher *Smad1* and lower levels of the BMP antagonist Gremlin (*GREM2*) (Pescatori *et al.*, 2007). The role and impact of these signaling cascades in the pathology of DMD is, however, not yet fully understood.

1.4 Overview of DMD therapy based on TGF-β inhibition

As described above, TGF-β signaling controls various aspects in DMD pathology. Therefore, research has been aimed at identifying and evaluating compounds that can inhibit signaling activity of TGF-β family members in dystrophic animal models. Some of these strategies have been tested in clinical trials (**Table 2**).

1.4.1 TGF-β antagonists

Losartan is an angiotensin II type 1 receptor blocker that originally was identified as an antihypertension drug, which antagonizes TGF-β signaling via repression of TGF-β and the expression of its target genes, such as connective tissue growth factor or collagen, thus improving extracellular matrix remodeling (Cohn *et al.*, 2007). Its therapeutic potential via attenuation of TGF-β signaling in various conditions/diseases such as sarcopenia, chronic renal disease, cardiomyopathies and Marfan syndrome has been described (Lavoie *et al.*, 2005; Habashi *et al.*, 2006; Cohn *et al.*, 2007; Burks *et al.*, 2011). Cohn *et al* showed that a 6-months administration of losartan in *mdx* mice attenuated TGF-β signaling, decreased skeletal muscle fibrosis and improved muscle regeneration, suggesting its potential for amelioration of the DMD pathology (Cohn *et al.*, 2007). However, more recent studies described that the improvement in muscle strength or function was only observed in a short-term treatment (2 months), but not in longer treatment duration of 6, 9 months and 2 years (Bish *et al.*, 2011; Nelson *et al.*, 2011; Spurney *et al.*, 2011). Nevertheless, these studies showed improvement in cardiac and/or respiratory function of the *mdx* mice, which suggest that this treatment strategy might still be beneficial and warrants an optimization of the dosing regimen.

Antibody targeting TGF-β has been tested in several preclinical studies as well. Andreetta *et al* treated *mdx* mice with intraperitoneal injection of a neutralizing anti TGF-β1 antibody

from 6-12 weeks of age and showed significantly reduced diaphragm fibrosis (Andreetta *et al.*, 2006). This effect was accompanied by decrease in *TGF-β1* mRNA and protein expression; although no obvious effect on muscle regeneration was observed (Andreetta *et al.*, 2006). One less encouraging finding was that this treatment increased $CD4^+$ lymphocytes, which suggests that inflammation is induced upon treatment with this antibody. However, this is in contrary to a more recent study, in which neutralizing antibodies targeting all three TGF-β isoforms were administered for 2 weeks in both 2- and 9-months old *mdx* mice (Nelson *et al.*, 2011). The treated mice improved the respiratory function and forelimb grip strength, but there was no evidence of increased *CD4* transcript expression upon treatment, indicating that this strategy may not lead to skeletal muscle inflammation. With such discrepancies, it is important to further evaluate any immunological consequences arising from TGF-β inhibition.

Decorin, a small leucine-rich extracellular proteoglycan, is an endogenous binding partner of TGF-β and able to inhibit its activity. Suppression of decorin production accelerates terminal differentiation of C2C12 myoblasts and significantly decreases the sensitivity to TGF-β and myostatin-dependent inhibition of myogenesis (Riquelme *et al.*, 2001). Miura *et al* reported the ability of decorin to bind and trap myostatin, in addition to TGF-β in collagen matrix, preventing interaction with its membrane-bound receptors and thus the inhibitory effect of myostatin on myoblast proliferation (Miura *et al.*, 2006). Decorin also prevented TGF-β-induced differentiation of myogenic cells into fibrotic cells in injured regenerating skeletal muscle (Li *et al.*, 2004). Systemic administration of decorin in *mdx* mice reduced collagen I expression, especially in the diaphragm, the muscle showing highest level of fibrosis (Gosselin *et al.*, 2004). Furthermore, decorin gene transfer promotes muscle cell differentiation and regeneration in *mdx* mice (Li *et al.*, 2007).

1.4.2 Myostatin antagonists

Monoclonal antibody-mediated myostatin blockade has shown beneficial effects in *mdx* mice, suggesting a promising therapeutic approach for DMD patients. One antibody produced against the C-terminal part of myostatin (MYO-029) resulted in increased skeletal muscle mass and strength (Bogdanovich *et al.*, 2002; Whittemore *et al.*, 2003). This has led to a Phase I/II clinical trial aimed at testing the safety profile of this molecule, which included 116 individuals with muscular dystrophies, including Becker muscular dystrophy, facioscapulohumeral dystrophy (FSHD), and limb-girdle muscular dystrophy (LGMD) (Wagner *et al.*, 2008). This study concluded that the antibody has a good safety and tolerability profile within the 24 weeks treatment period, but due to the short-term nature of the trial and small sample sizes, improvements in quantitative muscle strength and mass in the patients were not observed (**Table 2**). A small follow up study was performed by isolating a single muscle fiber from some of these patients to assess the cellular physiology. The contractile properties of either type I or IIa fibers were improved, in particular in the FSHD and LGMD patients (Krivickas *et al.*, 2009). Despite the lack of improvement in whole muscle size, strength and function in the first trial, this small study suggests that further investigation is required to better understand the molecular mechanism of myostatin antagonism in muscular dystrophy. Nevertheless, different variants of the anti-myostatin antibody are currently being developed, such as a mouse chimera of anti-human myostatin antibody. *Mdx* mice receiving this antibody variant had improved diaphragm functional capacity, although only when the treatment was initiated early, but unfortunately was ineffective at the later stage of the disease (Murphy *et al.*, 2010).

Table 2. Overview of therapies in trials [Source: clinicaltrials.gov]

Compound	Sponsor (Trial identifier)	Participants
Myostatin neutralizing antibody MYO-029	Wyeth (NCT00563810)	Healthy volunteers, intravenous infusion for 30 minutes
	Wyeth (NCT00104078)	Muscular dystrophy patients (BMD, FSHD, LGMD), intravenous infusions every 2 weeks for 6 months
Soluble Activin receptors (ACE-031, ActRIIB-IgG1)	Acceleron Pharma (NCT00755638)	Healthy postmenopausal women, single subcutaneous injection, placebo controlled
	Acceleron Pharma/ Shire (NCT00952887)	Healthy postmenopausal women, multiple-dose, placebo-controlled: two or three subcutaneous injections over a period of 1 month, or seven subcutaneous injections over a period of 3 months
	Acceleron/Shire (NCT01099761)	DMD patients receiving corticosteroids, multiple ascending-dose, placebo-controlled: seven subcutaneous injections every 2 weeks over a 12-week period, four subcutaneous injections every 4 weeks over a 12-week period
Ataluren (PTC124)	PTC Therapeutics/ Genzyme (NCT01247207)	Previous Ataluren trials participants to receive oral, 3X per day for 48 weeks
PRO051/ GSK2402968	Prosensa Therapeutics /GlaxoSmithKline (NCT01254019)	DMD boys, receiving placebo or a dose of 2OMePS oligos targeting exon 51 for 48 weeks
	Prosensa Therapeutics / GlaxoSmithKline (NCT01462292)	DMD boys receiving placebo or 2 doses of 2OMePS oligos targeting exon 51 for 24 weeks
	Prosensa Therapeutics / GlaxoSmithKline (NCT01128855)	Non-ambulant DMD boys receiving subcutaneous injections of placebo or 4 different doses of 2OMePS oligos targeting exon 51 for 24 weeks
PRO044	Prosensa Therapeutics (NCT01037309)	DMD boys receiving multiple doses of 2OMePS oligos targeting exon 44 via weekly subcutaneous or intravenous injections for 5 weeks
AVI-4658	AVI BioPharma (NCT00844597)	DMD boys receiving infused morpholino oligos targeting exon 51 at different doses for 12 weeks

Another strategy to inhibit myostatin function is via its propeptide, the amino-terminal part of synthesized myostatin, which is normally cleaved by BMP1/tolloid proteinases to give rise to a biologically active myostatin that can bind to its receptors (Wolfman *et al.*, 2003). The myostatin propeptide, however, is able to interact with myostatin ligand and inhibit the activity of myostatin by preventing receptor binding. The administration of a propeptide-based myostatin inhibitor that was resistant to cleavage by BMP1/tolloid proteinases showed positive results on various parameters in *mdx* mice such as muscle mass, endurance time on a rotarod,

	Phase/ Status	Outcome
	I Completed in April 2006	Good safety and tolerability
	I/II Completed in January 2007	Good safety and tolerability; no improvement in muscle strength; trend towards increase in muscle mass in some participants
	I Completed in July 2009	Sustained dose-dependent increases in lean mass and muscle volume; improved parameters of bone resorption and formation
	I/II Completed in February 2011	Good safety and tolerability; minor headache and irritation at the injury sites; two highest doses of the drug caused 3.3% increase in lean muscle mass and 5.1% increase in thigh muscle volume; increased markers for bone formation and decreased fat formation markers; a trend for improved grip strength
	II Terminated in 2011	Increase in muscle mass. Some participants experienced minor nosebleeds, gum bleeding, and/or small dilated blood vessels under the skin, which were fully resolved upon termination of the treatment
	III Recruiting	Not yet available
	III Recruiting	Not yet available
	II Recruiting	Not yet available
	I Ongoing but not recruiting	Not yet available
	I/II Ongoing, not recruiting	Not yet available
	I/II Completed	Safe and well-tolerated, successful dystrophin restoration

twitch and tetanic force, as well as serum creatine kinase levels, suggesting a reduction in muscle damage (Bogdanovich *et al.*, 2005; Qiao *et al.*, 2008; Matsakas *et al.*, 2009).

Downregulation of *myostatin* expression by siRNAs resulted in induced muscle growth in normal and cancer cachexia mice and in a mouse model of limb-girdle muscular dystrophy 1C, but this approach has not been tested in *mdx* mice (Liu *et al.*, 2008; Kawakami *et al.*, 2011). Another approach to specifically target myostatin expression is to modulate the normal splicing process using antisense oligonucleotides (AON). This strategy successfully induced exon skipping

and downregulated myostatin expression in myoblast culture. Two AON chemistries, namely 2'-O-methyl phosphorothioate (Kemaladewi *et al.*, 2011b) and octa guanidine-conjugated morpholino (Kang *et al.*, 2011) were tested, however, only the later one resulted in significant functional knockdown in *mdx* mice. In this study, knockdown and increase in muscle mass was only reported in the soleus muscle but was not observed in the extensor digitorum longus (EDL) muscle and, furthermore, the effect on muscle function was not described (Kang *et al.*, 2011).

1.4.3 Antagonists targeting multiple TGF-β ligands

ACVR2B is the primary type II receptor for activin, myostatin and GDF11. Soluble forms of ACVR2B have been generated, containing only the extracellular domain of the receptor fused to the soluble Fc domain of IgG protein, without the transmembrane and cytoplasmic kinase domains of the receptor. The resulting soluble ACVR2B compound still retains the ligand binding activity but is unable to exert signaling, thereby acting as a ligand trap and blocking ligand binding to endogenous receptors. This molecule, addressed as ActRIIB-Fc is useful to block actions of multiple ligands utilizing ACVR2B and has been explored in various DMD animal models. ActRIIB-Fc administration increased muscle mass by 32-61% in wild-type mice (Lee *et al.*, 2005). Preclinical studies in *mdx* mice have shown that intraperitoneal administration of ActRIIB-Fc for 3 months increased skeletal muscle mass and caused decreases in creatine kinase levels (Pistilli *et al.*, 2011). Adeno-associated virus (AAV)-mediated gene transfer of ActRIIB-Fc to the liver led to increased skeletal muscle mass and force production in the EDL as well as reduced creatine kinase levels (Morine *et al.*, 2010). Beneficial results on body mass and increases in force pulling tension were also observed upon 90 days of subcutaneous treatment with ActRIIB-Fc into *mdx* mice, and were further potentiated by NF-κB inhibitor (George *et al.*, 2011). Acceleron Pharma has completed studies in healthy volunteers with single- and multiple-dosing of this compound (ACE-031) (www.acceleronpharma.com/products/ace-031).

A phase II study in DMD patients was started to determine if ACE-031 is safe, well-tolerated and increases muscle mass in the patients. However, during the course of the trial, side effects occurred as some participants experienced minor nose- and gum-bleeding and/or small dilated blood vessels under the skin. Although these side effects stopped upon discontinuation of the drug, the trial was terminated. The mechanism of the bleeding has to be investigated and reported to the Food and Drug Administration (FDA) before it can be resumed (**Table 2**).

Dumonceaux *et al* used an alternative approach in *mdx* mice and administered AAV-carrying shRNAs for downregulation of the expression of endogenous *Acvr2b,* which resulted in improvement of muscle physiology. This study further evaluated the combination of dystrophin restoration and myostatin pathway inhibition, and showed significant improvement of tetanic and specific forces when both strategies were concurrently applied (Dumonceaux *et al.*, 2010).

Follistatin, a protein that acts as an endogenous antagonist of BMPs, myostatin and activin has also emerged as attractive therapeutic molecule. Transgenic expression of a follistatin-derived protein improved muscle function and regeneration and increased muscle mass in wild-type mice (Lee *et al.*, 2010; Zhu *et al.*, 2011) and *mdx* mice (Nakatani *et al.*, 2008). Furthermore, injection of follistatin-overexpressing myoblasts in immunodeficient *mdx* mice resulted in more efficient muscle regeneration (Zhu *et al.*, 2011). Importantly, the question whether long-term inhibition of myostatin/activin also results in improved muscle histology and function in *mdx* mice was addressed in another study where the effect of AAV-mediated follistatin overexpression was determined (Haidet *et al.*, 2008). After a single injection of the

viral vector, long-term improvement was seen on muscle size/weight and muscle force up to 180 days after the injection. In older *mdx* mice of 210 days, long-term overexpression of follistatin up to 560 days resulted in decreased creatine kinase levels, increased hindlimb grip strength and improvement of muscle histology (Haidet *et al.*, 2008).

1.4.4 BMP antagonists

The therapeutic approach targeting BMP signaling is less explored compared to myostatin and TGF-β. A study from our group compared three widely known BMP antagonists, namely dorsomorphin, LDN-193189 and Noggin *in vitro* (Shi *et al.*, 2011). The first two belong to small molecule inhibitors that target the kinase domain of BMP type I receptor, whereas Noggin prevents BMP binding to their receptors via direct interaction with the ligand. Noggin was found to be potent and specific to inhibit BMP signaling *in vitro* and enhanced myogenic differentiation of myoblasts. Adenoviral overexpression of *Noggin* in the *mdx/utrn*$^{+/-}$ mice increased myogenic regeneration markers *Myog* and *MyoD1*, as well as decreasing fibrotic/necrotic area, suggesting a potential beneficial effect of BMP antagonists in the dystrophic phenotype (Shi *et al.*, 2011). Interestingly, we did not observe any increase in fibrosis upon treatment with Noggin, as has been reported in damaged muscle of wild-type mice (Ono *et al.*, 2011). Future research into the role of BMP signaling during muscle regeneration and in DMD pathology should provide more insight to whether BMP antagonists can be considered as a potential DMD therapy.

1.4.5 Other compounds

Other compounds that do not specifically target TGF-β ligands but can inhibit TGF-β signaling have been evaluated in *mdx* mice. Halofuginone is a potent antifibrotic agent that suppresses collagen synthesis mediated by TGF-β signaling via inhibition of Smad3-activation (Granot *et al.*, 1993). In muscle cells, halofuginone inhibited Smad3 phosphorylation by inducing the association of non-phosphorylated Smad3 with Akt and MAPK/ERK, resulting in enhanced myotube fusion (Roffe *et al.*, 2010). In *mdx* mice, intraperitoneal administration of halofuginone reduced Smad3 phosphorylation levels and collagen deposition in limb and cardiac muscles, leading to improved cardiac function (Turgeman *et al.*, 2008). Suramin is an antiparasitic compound that is FDA approved (Taniguti *et al.*, 2011). Suramin decreased creatine kinase levels of exercised *mdx* mice and attenuated fibrosis in almost all muscles, except for cardiac muscles. These effects were likely to be achieved via prevention of TGF-β binding to its receptors (Coffey, Jr. *et al.*, 1987) or via inhibition of myostatin expression (Nozaki *et al.*, 2008). Finally, oral administration of Bowman-Birk inhibitor concentrate, a serine protease inhibitor in *mdx* mice resulted in increased mass, tetanic force and fiber size in the EDL muscle, in addition to decreased muscle damage parameters and reduced Smad activity. Interestingly, there was a decline in TGF-β1, but increased myostatin expression in the treated animals (Morris *et al.*, 2010).

1.5 Therapeutic approaches to correct the damaged *DMD* gene or compensate the lack of dystrophin

The therapeutic approaches based on TGF-β/myostatin pathway interference generally aim to improve muscle regeneration, whereas another group of therapeutic approaches directly target the lack of dystrophin in muscle fibers. These are achieved by upregulation of the dystrophin homologue utrophin, or introduction of a functional *DMD* gene either via viral

vectors or cells, and correction of the open reading frame, either via stop codon read through or antisense oligonucleotides (AON)-mediated exon skipping. These potential therapies will be discussed next.

1.5.1. Utrophin upregulation

Utrophin is the autosomal homologue of dystrophin. It has ~85% sequence similarity with dystrophin in the N- and C-terminal domains and ~35% in their spectrin-like repeats (Tinsley *et al.*, 1992; Winder *et al.*, 1995). Utrophin expression in the muscle is abundant during embryonic development, but in adult is restricted at the neuromuscular junction (Nguyen *et al.*, 1991). Furthermore, the utrophin levels decrease as dystrophin levels increase, suggesting that both proteins are coordinately regulated in a spatiotemporal manner during development (Tanaka *et al.*, 1991; Kleopa *et al.*, 2006). In the dystrophin-deficient muscle, utrophin is upregulated and also localized at the sarcolemma where it is able to bind components of dystrophin-associated protein complex (Matsumura *et al.*, 1992; Kleopa *et al.*, 2006). Thus, utrophin may be able to functionally compensate for the lack of dystrophin in DMD muscle.

The potential of utrophin was shown by transgenic overexpression of either truncated (Deconinck *et al.*, 1997; Grady *et al.*, 1997) or full length (Tinsley *et al.*, 1998) utrophin in *mdx* mice, which led to improvement of their phenotypes. On the contrary, mice without both dystrophin and utrophin alleles (*mdx/utrn*$^{-/-}$) exhibit a very severe phenotype and typically die at 2-3 months of age (Deconinck *et al.*, 1997; Grady *et al.*, 1997), whereas *mdx* mice with one utrophin allele (*mdx/utrn*$^{+/-}$) fall between the phenotypes of *mdx* and *mdx/utrn*$^{-/-}$ (Zhou *et al.*, 2008; van Putten *et al.*, 2012).

Efforts to find compounds that can upregulate utrophin expression are ongoing. High throughput screenings of chemical compounds libraries, utilizing a cell-based luciferase assay containing part of human utrophin A promoter, led to the discovery of SMTC1100 (also called 2-Arylbenzoxazoles or BMN-195) (Chancellor *et al.*, 2011; Tinsley *et al.*, 2011). In *mdx* mice, oral administration induced the expression of utrophin in the sarcolemma to an extent that improved body strength and endurance (Tinsley *et al.*, 2011). A less encouraging finding was that in a phase I clinical trial, the drug was undetectable in plasma of healthy volunteers after oral administration, suggesting that it may not be absorbed properly by humans. New formulations to improve the bioavailability are currently being tested.

An interesting approach, called drug repositioning, is to perform screenings with libraries of approved drugs and to explore whether they can be beneficial in new applications. This led to the identification of Nabumetone, a COX1/COX2 inhibitor that acts as anti inflammatory and has been used in osteoarthritis and rheumatoid arthritis and was able to increase utrophin levels *in vitro* (Moorwood *et al.*, 2011). While further preclinical testing in dystrophic mouse model is ongoing, nabumetone itself has been proven to be well-tolerated in humans. A recently described interaction of the human utrophin promoter with a homeobox protein Engrailed 1 (EN1) might hint at a transcriptional regulatory mechanism which can further serve as a means to regulate utrophin expression (Wang *et al.*, 2011a).

It was recently described that utrophin expression in juvenile mice is regulated by extracellular matrix protein biglycan (Amenta *et al.*, 2011), which binds to α-dystroglycan and α- and γ-sarcoglycan in the DGC (Bowe *et al.*, 2000; Rafii *et al.*, 2006). Biglycan also regulates the expression and sarcolemmal localization of other DGC constituents dystrobrevin, syntrophin and nNOS, thereby maintaining muscle integrity (Mercado *et al.*, 2006). Administration

of recombinant biglycan in *mdx* mice increased utrophin expression as well as other DGC components at the sarcolemma, and eventually ameliorated muscle pathology and improved motor function (Amenta *et al.*, 2011). Biglycan acts via an apparently unique mechanism that differs from chemical compounds described above. An optimized version of recombinant biglycan, called TVN-102, has been developed by Tivorsan Pharmaceuticals and designated as the lead clinical candidate (www.tivorsan.com).

1.5.2. Cell therapy

The idea behind cell-based therapies is to transplant donor cells that are able to fuse with the existing dystrophin-negative myofibers, provide the missing gene to replace dystrophin, contribute to the repair process and consequently improve muscle pathology. The donor cells can either be obtained from a normal individual (allograft), or from the patient himself (autograft), after they have been engineered to express dystrophin.

Several kinds of cells with myoregenerative capacity that are being studied include satellite cells and other muscle or non-muscle derived stem cells. Adult-derived myoblasts became the first obvious choice for cell transplantation, since they can be isolated from healthy donors and expanded *ex vivo* to achieve sufficient numbers of cells to be injected. Pioneering experiments in the *mdx* mice demonstrated the ability of these myoblasts to fuse and give rise to dystrophin-positive myofibers (Law *et al.*, 1988; Partridge *et al.*, 1989), which led to clinical studies in DMD patients (Law *et al.*, 1990; Gussoni *et al.*, 1992; Tremblay *et al.*, 1993; Mendell *et al.*, 1995). This approach has been largely unsuccessful due to relatively rapid cell death of the majority of the transplanted cells. Immune system rejection may only partly explain the failure of myoblast therapy, as even transplantation of allogenic myoblasts derived from monozygotic twin does not lead to long-term survival and yielded only few dystrophin positive fibers (less than 1.5% at 6 months after transplantation) (Tremblay *et al.*, 1993). A more frequent myoblast transfer regimes did not yield any significant improvement over the single transplant protocol, even though it was accompanied with immune suppressive drugs to prevent rejection (Mendell *et al.*, 1995).

In addition, the poor dissemination of the injected cells was a major problem encountered in this approach. Even after intramuscular injection, the injected cells stay within the injections site and did not migrate within muscles (Lipton and Schultz, 1979; Morgan *et al.*, 1987; Huard *et al.*, 1994). Many attempts have been made, including variation in cell numbers injected, modification of cell delivery method, strategies to control acute rejection and priming the donor cell prior to transplantation with heat-shock or fibrin gel expansion to overcome these limitations, but these studies resulted in limited improvement of myoblast transplantation (Skuk *et al.*, 2006a; Skuk *et al.*, 2006b; Skuk *et al.*, 2007; Skuk and Tremblay, 2011; Gerard *et al.*, 2011).

Interestingly, a small percentage of the transplanted cells are able to enter the satellite cell niche within the transplanted muscles and contribute to muscle regeneration (Blaveri *et al.*, 1999; Heslop *et al.*, 2001). Studies were then focused on isolation and identification of different cell populations that display myogenic properties because different populations may exhibit greater proliferative and/or differentiation potential with enhanced survival rates. This leads to identification of the so-called muscle-derived stem cells (Qu-Petersen *et al.*, 2002), $CD133^+$ (Benchaouir *et al.*, 2007), $PW1^+/Pax7^-$ interstitial cells (Mitchell *et al.*, 2010), side population (Asakura *et al.*, 2002) and $CD34^-$ cells (Pisani *et al.*, 2010). Thus far the precise origin of these different populations of myogenic cells and the extent to which they act as muscle stem cells during homeostasis are largely unclear. Furthermore, it is extremely difficult to prepare

pure/homogenous cell populations due to the irregular immunophenotypes of these cells. One cannot rule out the possibility that a single rare cell type that displays higher muscle regenerative capacity is present within these extremely heterogeneous cell populations, in which the present marker set fails to distinguish them.

As an alternative to muscle resident cells, studies also focus on stem cells that are found in other organs/tissues and able to differentiate into muscles, such as mesoangioblasts and pericytes. Both are blood vessel-associated stem cells and share various markers, although the mesoangioblasts were first described to be originated from embryonic (Minasi *et al.*, 2002) and the pericytes from postnatal (Dellavalle *et al.*, 2007) tissues. The pericytes that were isolated from non-muscle tissues, such as fat or bone, exhibited myogenic properties (Crisan *et al.*, 2008). Systemic administration of these perivascular cells resulted in the most significant proportion to reach skeletal muscles of all cell types tested thus far. Furthermore, the ability of these cells to contribute to skeletal muscle regeneration has been demonstrated in dystrophic mice, *i.e.* α-sarcoglycan-null (Sampaolesi *et al.*, 2003) and SCID/*mdx* (Crisan *et al.*, 2008). Intra-arterial injection of mesoangioblasts in dystrophin-deficient golden retrievers led to engraftment, yielding up to ~10% dystrophin positive fibers and functional improvement (Sampaolesi *et al.*, 2006).

Their therapeutic use was envisioned as they can easily be purified from convenient tissue sources and expanded in culture without significant loss of differentiation potential. A phase I clinical trial in which DMD patients will undergo several consecutive mesoangioblast transplantations from healthy family members has been initiated in Italy. The first results are anticipated in 2013 (Source: PPMD USA website).

1.5.3. Gene therapy

The gene therapy approach aims to introduce a functional *DMD* gene into muscle fibers, allowing production of dystrophin proteins. There are several ways to deliver genomic information, such as the use of viral vectors in which the viral gene is replaced by *DMD* cDNA and transduced to the target tissues, in particular skeletal muscle and heart. The abundance and poor accessibility of muscle tissues are the major challenges for this approach, as muscle tissue makes up to 40% of the human body and muscle fibers are surrounded by layers of connective tissues, which filter out most of the larger viral particles.

Adeno-associated viruses (AAV) are able to infect both dividing and non-dividing cells such as the post-mitotic muscle fibers (Podsakoff *et al.*, 1994). Furthermore, because they do not generally integrate to the host genomes, the risk of having insertional mutagenesis leading to potential activation of proto-oncogenes is small. However, the packaging capacity of the AAV is ~4.9 kb, far below the size of full-length *DMD* gene (2.5 Mb) or even the coding sequence (14 kb).

To circumvent this, mini- or microdystrophin have been designed, which are based on the finding that some BMD patients are lacking large parts of the central rod domain and yet they are mildly affected (England *et al.*, 1990; Matsumura *et al.*, 1994). Internally deleted dystrophins retain their N-terminal actin binding- and C-terminal domains, which are thought to contain most of the necessary regions for the roles of dystrophin in signaling, structural support and assembly of dystrophin-associated proteins at the cell membrane. While these two domains are indispensable, some of the spectrin-like repeats in the rod domains can be deleted without impairing the functionality (Harper *et al.*, 2002). An AAV vector could accommodate a microdystrophin construct containing the N-terminal actin binding domain, 4 (out of 24) spectrin-like repeat

domains, 3 (out of 4) hinge domains and a dystroglycan-docking half of the C-terminal domain, resulting in good transduction, improved muscle function and quality upon local and systemic administration in the *mdx* mice (Gregorevic *et al.*, 2004; Gregorevic *et al.*, 2006).

AAV comes in different serotypes, which all have different tissue tropisms (Halbert *et al.*, 2000). The serotype 6 for example, gives the highest transduction efficiency and stable gene expression in cardiac and skeletal muscles (Gregorevic *et al.*, 2004; Gregorevic *et al.*, 2006). Despite successful gene delivery in mouse models (Blankinship *et al.*, 2004; Riviere *et al.*, 2006), strong immune response to AAV capsid has been exhibited after intramuscular injection of AAV2 or AAV6 vectors carrying various transgenes in dogs and monkeys (Wang *et al.*, 2007; Mingozzi *et al.*, 2007; Wang *et al.*, 2011b). These hurdles may be overcome by combining two serotypes known for either the enhanced muscle transduction ability or a well-defined safety profile as chimeric, *i.e.* AAV 2/8 (Foster *et al.*, 2008). Furthermore, the use of muscle-restricted species-specific promoter and meticulous design of the mRNA sequence have shown to be beneficial (Foster *et al.*, 2008). Taking these considerations into accounts, Koo *et al* showed high and stable levels of microdystrophin in dystrophic canine model upon administration of AAV2/8 expressing optimized, canine-specific microdystrophin (Koo *et al.*, 2011). In addition, a chimeric of AAV1-AAV2, called AAV 2.5 was designed to benefit from muscle-transduction efficiency of AAV1 and the heparin binding of AAV2 facilitating the purification and ease the production (Mendell *et al.*, 2010; Bowles *et al.*, 2012). This strategy was shown to successfully deliver the vector transgene in 6 DMD patients with no vector-related adverse events (Bowles *et al.*, 2012).

Nonetheless, this approach resulted in weak dystrophin restoration in only 2 patients, suggesting that the vector dose is probably less effective than predicted and needs further optimization (Mendell *et al.*, 2010). In parallel, this trial reported an unexpected finding that a subset of patients was shown to have had T-lymphocyte response to dystrophin even before treatment and elevated over time. Since these epitopes differ from those expressed by the vector, it was suggested that such pre-existing immunity to dystrophin epitopes may be primed by the revertant myofibers, which were observed in these patients and are generally quite common in DMD patients (Mendell *et al.*, 2010). This raised some concerns whether dystrophin itself may be immunogenic in the dystrophin-deficient patients. The numbers of patients included were very small and control experiment, *e.g.* non-injected patients were lacking in this study, and therefore the results should be carefully interpreted. In parallel, other trials that successfully increase dystrophin have not reported any anti-dystrophin antibodies {Goemans; Cirak; see below}.

1.5.4. Stop codon read-through

Some aminoglycoside antibiotics, such as gentamicin, can incorporate a random amino acid at the site of premature termination codon (Burke and Mogg, 1985), thereby suppressing the effect of a nonsense mutation without affecting the remaining of the transcript. Theoretically, this approach would be beneficial for any DMD patients harboring nonsense mutations, which represent ~14% of the mutations (Aartsma-Rus *et al.*, 2009). However, the read-through efficiency somewhat depends on the type of stop codon mutation, either UGA, UAA or UAG and the flanking nucleotides, resulting in relatively high (up to 20%) but variable dystrophin levels restored in the *mdx* mice (Barton-Davis *et al.*, 1999; Howard *et al.*, 2004). Furthermore, different gentamicin isomers appear to have different read-through efficacies. In a commercial production, each batch contains a mix of different isomers with different ratios, which can have

influence on the efficacy (Dunant *et al.*, 2003). When the proper isomer was administered, gentamicin-treated *mdx* mice showed improved histology, increased dystrophin expression and recovery of some of the DGC components (De Luca *et al.*, 2008).

The initial clinical trial reported in 2001 involving two DMD and two BMD patients found no dystrophin restoration upon 2 weeks gentamicin treatment (Wagner *et al.*, 2001), which may be explained by the duration of treatment or the source of gentamicin. A subsequent trial reported dystrophin restoration in 3 out of 4 patients treated with gentamicin over two cycles, each for 6 days with a 7-week hiatus between dosing (Politano *et al.*, 2003). Their findings favoured an effect related to a greater readthrough permissiveness of the UGA stop codon, but the small sample size and the under representation of other type of mutations detracts from this conclusion. In the third trial, 14 days gentamicin treatment resulted in lower serum creatine kinase in patients with stop mutations, whereas in the patients with frame-shifting deletions the CK remained high after one month post-treatment (Malik *et al.*, 2010). A 6-month gentamicin treatment resulted in up to 15% dystrophin restoration in 3 out of 16 patients with stop mutations (Malik *et al.*, 2010). Although encouraging, chronic, long-term treatment with gentamicin might lead to irreversible nephrotoxicity. Therefore, an alternative compound with a better safety profile is needed.

A screening assay to identify other similar agents resulted in the identification of PTC-124 (Ataluren), a more selective, safer nonaminoglycoside suppressor with oral bioavailability (Hirawat *et al.*, 2007). The preclinical study in the *mdx* mice led to ~25% dystrophin restoration, which was accompanied by improved muscle strength and lower CK levels (Welch *et al.*, 2007). A subsequent trial confirmed that ataluren was well-tolerated by healthy volunteers and DMD patients. A 4 weeks treatment regime with different doses led to a modest increase in dystrophin levels (11% increase in 65% of patients). In a subsequent phase II/III trial in which patients were treated with two doses or placebo for 48 weeks, there was a difference of 29.7 meters in the average change in 6MWD when comparing the placebo- with low-dose ataluren treatment, but not with the high dose, suggesting a bell-shaped dose response curve (http://www.mda.org/research/view_ctrial.aspx?id=214). Furthermore, patients receiving ataluren experienced significantly slower disease progression and positive trends in muscle function. Due to these findings, follow-up extension studies have been reinitiated in the US and will soon be reinitiated elsewhere.

1.5.5. Exon skipping

The exon skipping approach is based upon the existence of a DMD-related allelic disorder, Becker muscular dystrophy (BMD), which is caused by mutations that retain the open reading frame of the *DMD* transcript and create a shorter, but partially functional dystrophin. Muscular dystrophy severity is variable in BMD patients. It is typically much milder than DMD with a normal life-span, but it may range from DMD-like to virtually asymptomatic (Monaco *et al.*, 1988). Exon skipping aims to reframe the mutated *DMD* transcript by interfering with the splicing process and permitting translation to a Becker-like dystrophin. It involves the uptake of antisense oligonucleotides (AON), short RNA molecules that hybridize to the splice site of a target exon during pre-mRNA splicing, precluding their recognition by the splicing machinery and as such resulting in the skipping of the targeted exon (Spitali and Aartsma-Rus, 2012) (**Figure 1.5**).

The first *DMD* exon skipping was achieved for exon 19 in transformed lymphoblastoid cells (Pramono *et al.*, 1996), followed by exon 23 in *mdx*-derived myoblasts (Dunckley *et al.*,

1998; Wilton *et al.*, 1999). The broad therapeutic applicability of this strategy was shown upon achieving targeted exon skipping of in several patient-derived cells, leading to reframed transcript and restoration of dystrophin protein (van Deutekom *et al.*, 2001; Aartsma-Rus *et al.*, 2002; Aartsma-Rus *et al.*, 2003; Aartsma-Rus *et al.*, 2004).

These successful *in vitro* studies were followed by exon skipping in *mdx* mice (Reissmann *et al.*, 2001) and humanized DMD (hDMD) harboring full-length human DMD gene (Bremmer-Bout *et al.*, 2004; 't Hoen *et al.*, 2008) upon intramuscular injection. Systemic administrations have been proven to be feasible to induce exon 23 skipping in *mdx* mice (Lu *et al.*, 2005) and exons 6 and 8 skipping in several dystrophin-deficient dogs such as the golden retriever

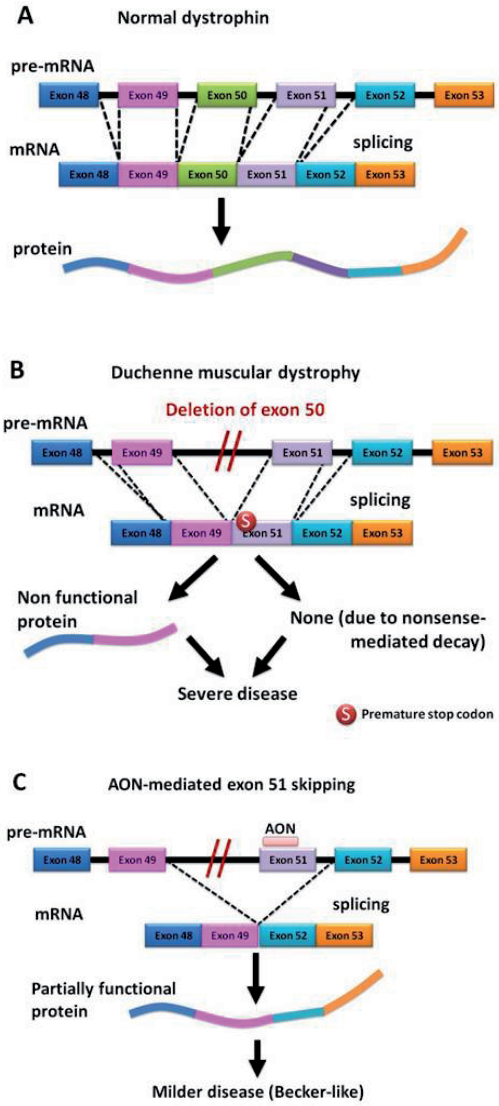

Figure 1.5. AON-mediated exon skipping to reframe the mutated DMD gene. Normal dystrophin pre-messenger RNA (pre-mRNA) undergoes normal splicing and results in normal dystrophin mRNA and protein (A). Note that only internal segment (exon 48-53) is shown for clarity purpose. The absence of exon 50 in the dystrophin gene leads to an out-of-frame mRNA creating a premature stop codon in exon 51, which either induces transcript clearance by means of nonsense-mediated mRNA decay or leads to the formation of a truncated, nonfunctional protein (B). The introduction of synthetic antisense oligonucleotides (AON) interferes with splicing signals and hides the targeted exon from splicing machinery. Together with its flanking introns, the hidden exon will be spliced out, leading to correction of the open reading and allowing the production of internally deleted, partly functional dystrophin protein (C).

muscular dystrophy (GRMD) (Yokota *et al.*, 2009) and canine X-linked muscular dystrophy ($CXMD_J$) (Yokota *et al.*, 2011).

A limitation of this approach is its inapplicability to patients with mutations in the domains binding to either the actin cytoskeletion or the extracellular matrix, because these domains are essential for dystrophin function. Furthermore, due to the mutation-specific nature, different exons need to targeted, which may necessitate extensive optimization of the therapy for each patient. The largest group of patients, comprising ~13% of all patients, would benefit from exon 51 skipping, which is the first focus of clinical development of this approach.

In addition to the diversity of AONs required to treat patients with different mutations, the bioavailability of AONs is a major concern. Various chemistry modifications of the AONs to promote their stability, uptake and extent to which they induce exon skipping are being explored (Lu *et al.*, 2005; Yin *et al.*, 2010; Yin *et al.*, 2011a; Yin *et al.*, 2011b). Two trials involving local administration of two different chemistries, namely 2'-*O*-methyl phosphorothioate modifications (2OMePS, PRO051/GSK2402968), and phosphorodiamidate morpholino oligomers (PMO, AVI-4658) were conducted. Successful dystrophin restoration after intramuscular injection of AON targeting *DMD* exon 51 were achieved with both compounds and no serious adverse effects were found (van Deutekom *et al.*, 2007; Kinali *et al.*, 2009).

The subsequent clinical trials with systemic administrations showed also encouraging findings. PRO051 was administered at escalating doses of 0.5 to 6 mg/kg in 12 DMD boys subcutaneously for 5 weeks. All doses were well tolerated and led to a dose-dependent increase of dystrophin expression. Furthermore, in an open label extension study where the highest dose of PRO051 was given, some participants showed increased walking distances in the 6 minutes walking test with average improvement of 35.2 ± 28.7 m (Goemans *et al.*, 2011). A phase II systemic administration trial with AVI-4658 has also been completed. Here, 19 patients received intravenous infusions of the oligos at the dose of 0.5 to 20 mg/kg or placebo for 12 weeks. Such dosing regimen was also well-tolerated and dystrophin was restored over pre-treatment levels in 7 patients at higher doses. In particular, 3 patients responded very well with increased dystrophin levels up to 18% in up to 55% of fibers (Cirak *et al.*, 2011). Furthermore, inflammatory infiltrates were reduced in patients receiving the two highest doses and increased sarcolemmal expression of neuronal nitric oxide synthase (NOS) was observed in the patients with the highest percentage of dystrophin restoration (Cirak *et al.*, 2011).

With the promising results from these trials, the exon skipping approach is considered to be the closest to a therapeutic application. Several follow up trials are currently ongoing worldwide, *i.e.* the PMO chemistry that is sponsored by AVI BioPharma and the 2OMePS chemistry by Prosensa Therapeutics and its partner GlaxoSmithKline (GSK).

The placebo-controlled multicenter trials by GSK/Prosensa include a phase III study where 6mg/kg/week of the compound was administered (NCT01254019), a phase II/III study assessing two dosing regiments of 3 mg/kg/week or 6mg/kg/week (NCT01462292), and a phase II study comparing 6mg/kg of the drug given once weekly or twice weekly (NCT01153932). In addition, a phase I/II trial in non-ambulant patients is ongoing (NCT01128855), which will provide the crucial answer whether AONs can be successfully applied in later stages of the disease. Furthermore, a phase I/II trial assessing the safety of PRO044, a 2OMePS AONs targeting exon 44, which will potentially benefit ~6% of patient population is also ongoing and the results are expected in the end of 2012 (NCT01037309).

In parallel, research is ongoing to improve the current chemistry. The uptake in the heart muscle has proved to be challenging but will be important. The demand for a stable sarcolemma is high for the myocardium because of the constant pumping activity of the heart. As a result, cardiomyopathy and/or heart arrhythmia are regularly seen in patients. The uncharged PMO chemistry can be conjugated with cell-penetrating peptides (PPMO), which are positively charged. Depending on the sequences of the peptides, these PPMOs have resulted in dystrophin restoration in numerous tissues including in the cardiac tissue in *mdx* mice and improved the survival of the severely affected *mdx/utrn*$^{-/-}$ mice (Yin *et al.*, 2010; Goyenvalle *et al.*, 2010; Yin *et al.*, 2011a; Yin *et al.*, 2011b). However, the reported toxicity of this new generation oligos presents a problem and remains to be resolved before they can be tested in a trial in human.

Finally, the generation of hDMD mouse facilitates the screening of human-specific oligo sequences (Bremmer-Bout *et al.*, 2004; 't Hoen *et al.*, 2008; Heemskerk *et al.*, 2009; Wu *et al.*, 2011). Although the hDMD mice show no phenotype, it bridges parts of the regulatory requirements for AONs to be met as human specific AONs generally do not work in mice due to sequence differences in the target sites.

1.6. The scope of this thesis

There are several aspects controlling the severity of dystrophic pathology in DMD. The major one is the absence of the vital muscle structural protein dystrophin. On the other side, elevated and continual activation of TGF-β signaling affects muscle regeneration and aggravates the disease. More than 25 years after the discovery of *DMD* gene, there are several promising therapeutic approaches to tackle different facets of the disease, including the exon skipping-mediated dystrophin restoration and pharmacological interference of the TGF-β pathway.

The first part of this thesis aims to better understand the molecular mechanism behind the signaling of TGF-β family member, in particular myostatin, in muscle. A new modulator of myostatin, Cripto, which is expressed in myoblasts but not in other cells, was identified. Cripto is a co-receptor facilitating myostatin-, an antagonist for activin-, but dispensable for TGF-β signaling in myoblasts, providing a potentially unique cell-type control mechanism for these three ligands in skeletal muscle (**Chapter 2**).

This thesis also presents a view that a combination therapy targeting multiple parts of the pathology might be a more ideal and holistic way to tackle a complicated disease like DMD. The AON-mediated exon skipping was introduced to the wide array of TGF-β-based therapy, first by interfering with the ligand expression (**Chapter 3**). In contrast to the application of AON to regain the function of dystrophin, the AONs were used to disrupt the open reading frame of myostatin and downregulate its expression, leading to a decrease the total level of myostatin available to exert its function.

In the next chapter, type I receptors ALK4/ALK5 necessary for TGF-β/myostatin/activin signaling were targeted (**Chapter 4**). Here, the strategy lays on truncating several domains important for the function of these receptors using AONs, in which the open reading frame was either maintained or disrupted. Treated *mdx* mice were analysed for any effects of ALK4 and ALK5 AONs in regulating myogenic regeneration and fibrosis both *in vitro* and *in vivo*.

Several advantages and disadvantages of using AON to modify the TGF-β pathway, either by modulating the ligand expression or the receptor functions can be envisioned and will be addressed in the general discussion (**Chapter 5**). The potential advantages of using AON as a

combination therapy to correct genetic damage and improve muscle quality will be discussed. Furthermore, some comparisons with other strategies to inhibit TGF-β family signaling, including myostatin and activin for muscle and non-muscle diseases will be made and lessons to be learnt from these strategies will be outlined.

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