# CHAPTER 5

## GENERAL DISCUSSION

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## 5.1. The challenges in developing DMD therapies

DMD is a monogenic disease with a complex multilayered pathophysiology. The causative *DMD* gene was mapped to Xp21 about 27 years ago (Ray *et al.*, 1985; Monaco *et al.*, 1986; Kunkel *et al.*, 1986; Koenig *et al.*, 1987) and is the largest gene in the human genome. The size makes *DMD* gene prone to mutations, in which deletions and point mutations were encountered as the most and the least frequent types, respectively (den Dunnen *et al.*, 1987; Davies *et al.*, 1988; den Dunnen *et al.*, 1989; Aartsma-Rus *et al.*, 2006). Moreover, some regions in the *DMD* gene, usually called the hotspot regions seem to be more susceptible to mutations, as the majority of the deletions and duplications cluster here (Liechti-Gallati *et al.*, 1989; den Dunnen *et al.*, 1989; Beggs *et al.*, 1990).

Following the discovery of the causative gene and despite extensive research for a variety of different approaches, there is no clinically applicable therapy yet. A lot of DMD patients are treated with steroids, which have an effect on stabilizing or even improving muscle strength. The downside of steroid treatment is that they may have various side effects, which may be reasons for discontinuation. The side effects range from short-term effects like weight gain and mood changes to long-term effects such as growth suppression, development of cataracts, spinal deformation and stomach irritation (Manzur *et al.*, 2008). The molecular mechanism how steroids trigger such side effects and how these drugs slow down the dystrophic process are unknown.

There are several explanations for the lack of efficient therapies for DMD. The abundance of muscle tissues to target, including heart, diaphragm and skeletal muscles, requires the drugs to be delivered systemically. Cardiac muscles, in particular, are difficult to target with the therapeutic strategies that are currently in development (Heemskerk *et al.*, 2009; Yin *et al.*, 2010a; Yin *et al.*, 2010b; Yin *et al.*, 2011a; Yin *et al.*, 2011b; Lai and Duan, 2012; Zhang and Duan, 2012). Furthermore, cardiomyopathy is usually observed in BMD patients as well, suggesting that factors other than the lack of dystrophin may contribute to cardiac dysfunction and dilation in DMD (Bushby *et al.*, 1993; Schade van Westrum *et al.*, 2011). Previous findings regarding the progression of dilated cardiomyopathy in the non-dystrophic heart suggest that progressive myocyte loss, fibrosis, hypertrophy and myocyte slippage involve in the left ventricle dilation (Beltrami *et al.*, 1995; Dobaczewski and Frangogiannis, 2009). The degree to which these processes contribute to DMD cardiomyopathy is currently unknown.

With regard to the skeletal muscles, it is currently unclear whether there is a minimum threshold of dystrophin restoration to reach functional improvement. Comparative studies of mice with various dystrophin levels in *mdx* and *mdx/utrn* negative background suggested that 5-10% of normal dystrophin levels, which are relatively marginal, might already be sufficient to improve life span, muscle histopathology and motor function, but higher levels would be required for a better protection from exercise-induced damage (Li *et al.*, 2010; van Putten *et al.*, 2012a; van Putten *et al.*, 2012b).

Another obstacle in the development of DMD therapies is the use of mouse models to assess the therapeutic efficacy. *Mdx* mice are widely used to study the outcome of therapeutic interventions. However, these mice have normal life-span and demonstrate a much less severe phenotype than DMD patients. Therefore, more severe mouse models are used, such as the *mdx/utrn*$^{-/-}$ (Deconinck *et al.*, 1997) or *mdx/mTR* mice (Sacco *et al.*, 2010), which may reflect the human disease better.

Furthermore, it is important to deal with every aspect that accumulatively exacerbates the disease, including impaired muscle regeneration, ongoing inflammation and excessive fibrosis. There are multiple players in these processes, including different cell types and involvement

of different signaling pathways (**Chapter 1**). As there is a continuous cycle of de-/regeneration in the dystrophic muscle, a lifelong treatment to restore dystrophin function is most likely needed. Moreover, DMD progresses relatively rapidly, thus therapeutic interventions need to be administered as early as possible before significant muscle loss occurs. Muscle biopsies of pre-symptomatic patients (<2 years) already showed dystrophic molecular signature, evident from altered expression of genes involved in inflammatory response, extracellular matrix remodeling and muscle regeneration (Pescatori *et al.*, 2007). At the average age of diagnosis, 4-5 years, the muscle wasting already occurs (Bushby *et al.*, 1999). Neonatal screening, which is based on the measurement of serum creatine kinase levels, would allow the affected boys to be identified as early as possible so that they can be treated before significant muscle pathology develops.

Several therapeutic strategies are currently in development, such as efforts to introduce functional dystrophin via viral vectors or cell transplantation, bypass the mutation and restore the open reading frame (stop codon read-through or exon skipping) or upregulate the homologous protein utrophin. Amongst them, antisense oligonucleotide (AON)-mediated exon skipping to reframe the mutated *DMD* gene is considered to be the most advanced, as encouraging results have been reported from ongoing multicenter trials (van Deutekom *et al.*, 2007; Kinali *et al.*, 2009; Cirak *et al.*, 2011; Goemans *et al.*, 2011).

Nevertheless, this specific approach also comes with several challenges to tackle. Theoretically, 83% of DMD patients could be treated with AONs, but an AON targeting a specific exon is only beneficial for a specific subset of mutations. Exon 51 skipping, the most advanced in the trials, would benefit ~13% of all patients, and skipping in the 10 most relevant exons would only be beneficial for around 40-50% of patients (Aartsma-Rus *et al.*, 2009). Clinical development programs for exon skipping AONs have been prioritized according to the number of patients that could potentially be treated (please refer to www.prosensa.eu for the development pipeline of DMD exon skipping). Multi-exon 45-55 skipping in the hostpot mutation region has been suggested as worthwhile for investigation (Beroud *et al.*, 2007), as not only it would increase the number of eligible patients to ~63% of patients, it would also create a deletion associated with a mild phenotype (Ferreiro *et al.*, 2009; Helderman-van den Enden AT *et al.*, 2010). Although conceptually sound and despite extensive research, such a multi-exon skipping approach remains refractory to practical application (van Vliet *et al.*, 2008; Goyenvalle *et al.*, 2012).

Finally, dystrophin restoration strategies rely on the availability of muscle fibers, which express the *DMD* gene and where the mutated *DMD* gene can be "skipped". Dystrophin is produced by muscle fibers, but not by fibrotic and fat tissues which are accumulated at the advanced stage of the disease, and is important for muscle fiber function. Therefore, any therapeutic intervention that can improve muscle quality, either by reducing fibrotic and fat deposition or improve muscle regeneration would serve as an attractive adjunctive, as more muscle fibers will become amenable to exon skipping. Such therapies to combat the secondary disease pathology include those based on intervention of TGF-β/myostatin signaling pathway.

## 5.2. The challenges in developing DMD therapy based on TGF-β family signaling pathway

Signaling pathways of the TGF-β family members play prominent roles in the diverse aspects of DMD pathology, such as impaired muscle regeneration, excess fibrosis and ongoing inflammation (**Chapter 1**). The results obtained with the different antagonists of these

pathways in *mdx* mice show their promise as a therapeutic approach for DMD. The studies on hypermuscular myostatin knock out animals opened a spectrum of myostatin-based treatment strategies for DMD. However, one should realize that extrapolating the effects of selective genetic ablation of TGF-β family ligands, such as myostatin, may not be the same as those achieved upon postnatal inhibition of such proteins. In addition, several studies on hypermuscular myostatin knockout mice revealed that the increase in muscle mass and fiber size was not accompanied by an increase in specific force (Amthor *et al.*, 2007; Qaisar *et al.*, 2011). This might be partly due to larger myonuclear domains observed in myostatin knockout fast muscle fibers (Qaisar *et al.*, 2011) and to small, brittle and hypocellular tendons observed in these mice (Mendias *et al.*, 2008). The method to measure muscle strength, ranging from grip strength to single fiber contractile capacity, also complicates the interpretation and comparability across different studies. Nevertheless, the current consensus in the field is that excessive muscle growth due to complete myostatin absence may not be solely beneficial because it compromises the ability to generate force. A partial inhibitory approach, such as the use of AON to decrease the expression of myostatin transcript (**Chapter 3**) or the receptors (**Chapter 4**) might be might be more favourable and warrants further investigation.

The next challenges involve how to direct this type of intervention to muscle. Since its discovery in 1997, it is now firmly established that myostatin is synthesized mainly by skeletal muscle but also by fibrotic and adipose tissue, circulates in the blood and acts in a concentration-dependent manner as a negative regulator of muscle growth. The way myostatin reconciles this type of local control with the fact that it circulates systemically in the body is therefore intriguing.

Myostatin is secreted in an inactive form, in which the active portion of the molecule remains non-covalently bound to and inhibited by its propeptide, rendering the latency. One mechanism by which myostatin is activated from this latent state is via cleavage of the propeptide by members of the BMP-1/tolloid family of metalloproteases (Wolfman *et al.*, 2003). In addition to the propeptide, a number of proteins have also been identified that are capable of binding myostatin, such as FLRG (follistatin-related gene) and GASP-1 (growth and differentiation factor-associated serum protein-1). These proteins were found to be complexed to myostatin in mouse and human serum and appear to be involved in regulating the activity of a proteinase that cleaves the propeptide to activate latency (Hill *et al.*, 2002; Hill *et al.*, 2003). Genetic experiments to eliminate to the functions of GASP-1 would be needed to dissect its role in controlling the overall level of active myostatin. FLRG knockout mice showed enhanced insulin sensitivity and altered visceral fat deposition to subcutaneous depots, without any alteration in total body weight and fiber, suggesting that increased myostatin bioactivity due to abrogation of FLRG results in lipid homeostasis. Nevertheless, it is clear that another modulatory mechanism, beyond simply regulation of myostatin expression, reflect the actual level of myostatin signaling in different tissues.

An alternative mechanism for achieving specificity is via selective utilization of co-receptors. **Chapter 2** reveals the presence of myostatin co-receptor Cripto in myoblasts, but not in non-myogenic cells such as muscle fibroblasts. Together with ALK4, Cripto regulates myostatin signaling in myoblasts. Furthermore, it delineates the signaling specificity of TGF-β family members in myoblasts, as it is able to act as enhancer and antagonist for myostatin and activin signaling, respectively, but dispensable for TGF-β signaling. The spatiotemporal

expression of Cripto as well as its function in adult skeletal muscle remain to be elucidated. Nevertheless, the specific interaction of this receptor with myostatin suggests that Cripto may become a suitable candidate for muscle-targeted myostatin-based therapy with lower chance of on-target side effects.

Interfering with the function of multiple TGF-β members, such as by targeting their common receptors might yield more striking effects, but perhaps has a greater risk of side effects in tissues other than skeletal muscle upon systemic administration. The recent preliminary result of the clinical trials with Acceleron Pharma's ACE-031 is a good example in this respect. The preclinical studies in *mdx* mice led to normalization of muscle force and the further phase I clinical trial in healthy volunteers led to increase in muscle mass as well as reduced fat tissues with good tolerability in both single and multiple doses regimes. The continuation of this trial in DMD patients led to increase in lean body mass and trend towards maintenance of muscle function in 6-minutes walking test in some participants. However, unexpected nose and gum bleeding occurred in some participants and resolved upon withdrawal of the drug. This caused suspension of the trial and requires further investigation of the mechanisms underlying these unexpected side effects.

Small molecule kinase inhibitors can also be employed to inhibit the TGF-β family signaling pathway. They are able to block the kinase activity of the TGF-β type I receptor, thereby preventing phosphorylation of downstream Smads (Laping *et al.*, 2002; Peng *et al.*, 2005). These small molecule inhibitors target the kinase domain of the receptors. As a consequence, they tend to also target the other type I receptors with similar conformation (Inman *et al.*, 2002; Yingling *et al.*, 2004). Moreover, a recent study showed that different small molecule ALK5 inhibitors, including LY364947 and SB431542, also inhibit activity of other protein kinases, such as casein kinase 1, receptor interacting protein kinase 2 and p38 MAPK (Vogt *et al.*, 2011). In contrast to their therapeutic potential in cancer or other diseases, these inhibitors have not been widely assessed in the context of muscle disease. A study in *Xenopus* model showed that SB431542, an inhibitor originally designed to inhibit ALK4 and ALK5 was able to induce muscle hypertrophy, but decreased the specific force (Watt *et al.*, 2010).

Our experiments showed that LY364947 could potently inhibit TGF-β- and myostatin-induced luciferase activity *in vitro*, but was unable to differentiate between ALK4- or ALK5-mediated myostatin signaling, showing its poor specificity (**Figures 5.1** and **5.2**). Also it has been described that these compounds are relatively instable and therefore require more frequent administrations (Kano *et al.*, 2009). We treated *mdx* mice daily with the small inhibitor LY364947 but did not observe any changes in the myogenic and fibrotic markers, nor in creatine kinase levels (**Figure 5.3)**. Although not elucidated further, it is possible that the bioavailability of this molecule was low and may require higher doses or more chronic treatment. On the other hand, such chronic treatment regimes might lead to a feedback-loop mechanism via overcompensation by other nodes of the pathway, thus rendering its effects less effective (Miyazono, 2000; Rebbapragada *et al.*, 2003; Korupolu *et al.*, 2008).

The complexity of TGF-β signaling results in challenges if one considers inhibition of these signaling cascades as a potential therapeutic approach. A better understanding of how TGF-β members act in muscle biology is still needed, and thus more basic research is required.

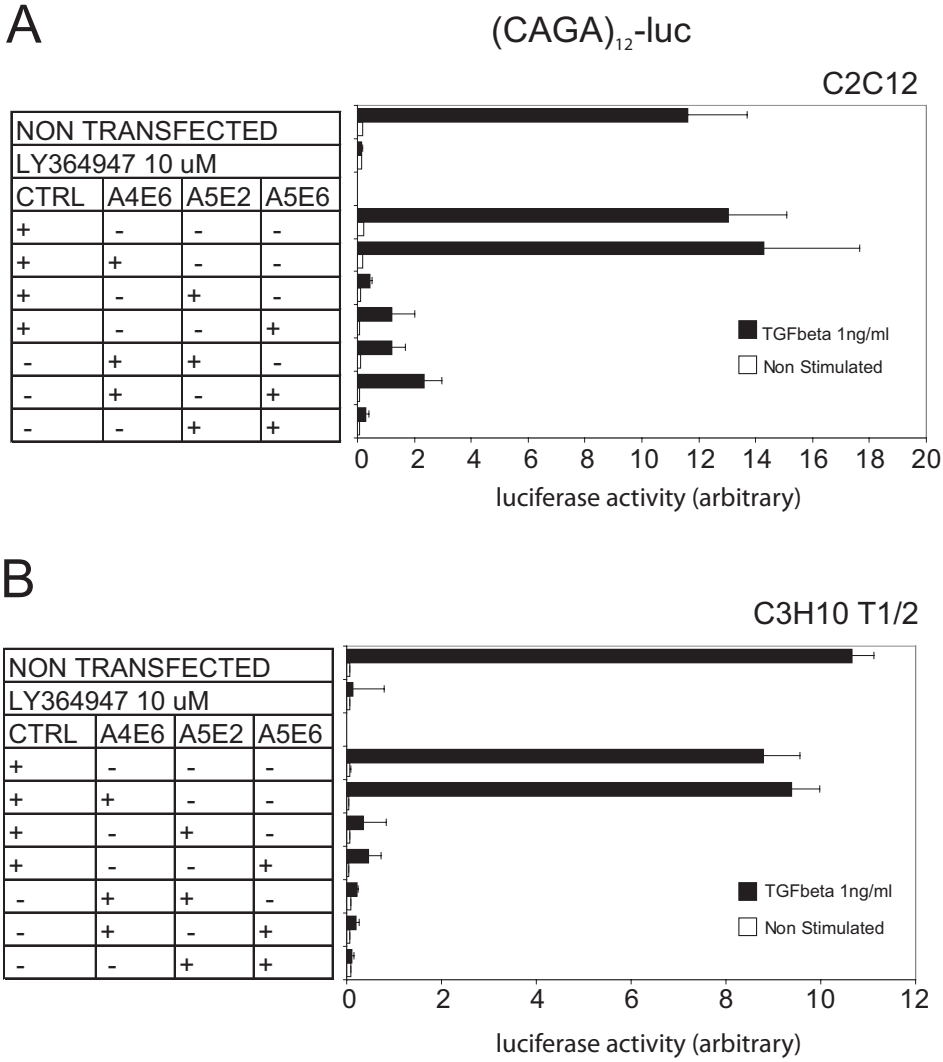


**Figure 5.1. TGF-β-induced signaling can be potently inhibited by LY364947 and ALK5 AONs.** Mouse myoblast C2C12 (A) and mesenchymal stem cells C3H10 T1/2 (B) were transfected with $(CAGA)_{12}$-Luc and Renilla-Luc constructs, with either control, ALK4 (A4E6) or ALK5 (A5E2 or A5E6) AONs. 24 hours later, the cells were serum starved overnight. LY364947 was added to the medium 30 minutes prior to 8 hours TGF-β stimulation. The experiments were conducted in triplicate and the average measurement was plotted. Error bars represent standard deviation of the triplicates.

A

$(CAGA)_{12}$-luc

C2C12

| NON TRANSFECTED | | | |
|---|---|---|---|
| LY364947 10 uM | | | |
| CTRL | A4E6 | A5E2 | A5E6 |
| + | - | - | - |
| + | + | - | - |
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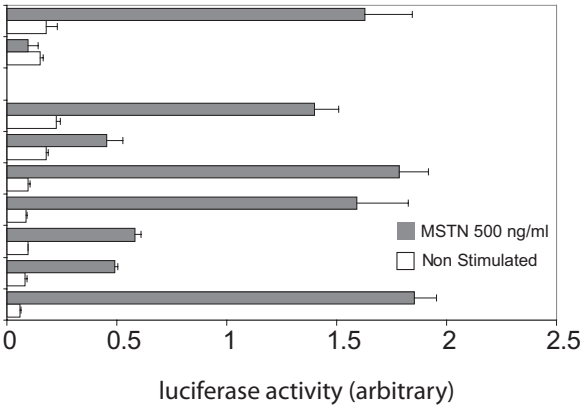


B

$(CAGA)_{12}$-luc

C3H10 T1/2

| NON TRANSFECTED | | | |
|---|---|---|---|
| LY364947 10 uM | | | |
| CTRL | A4E6 | A5E2 | A5E6 |
| + | - | - | - |
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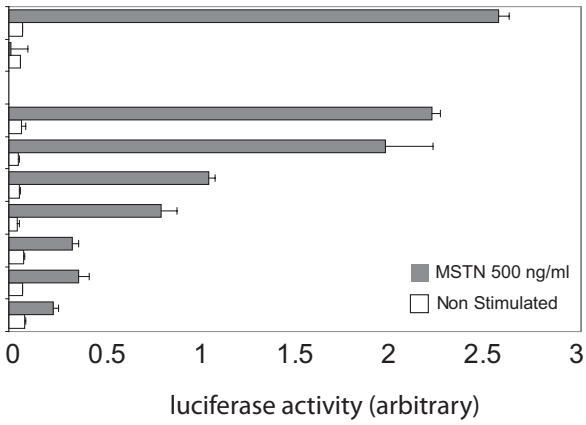


**Figure 5.2. LY364947 failed to distinguish cell-type utilization of type I receptors by myostatin.** Mouse myoblast C2C12 (A) and mesenchymal stem cells C3H10 T1/2 (B) were transfected and treated as described in Figure 5.1, except that the stimulant was myostatin instead of TGF-β. Note that in myoblasts (A), ALK4 inhibition by AON can abrogate myostatin signaling, whereas ALK5 can not. In contrast, myostatin signaling in C3H10 T1/2 was inhibited by ALK5 knockdown, but not by ALK4 knockdown. This differential mechanism was not observed in LY364947-treated cells, suggesting that at least 2 type I receptors, ALK4 and ALK5, were targeted by this approach.

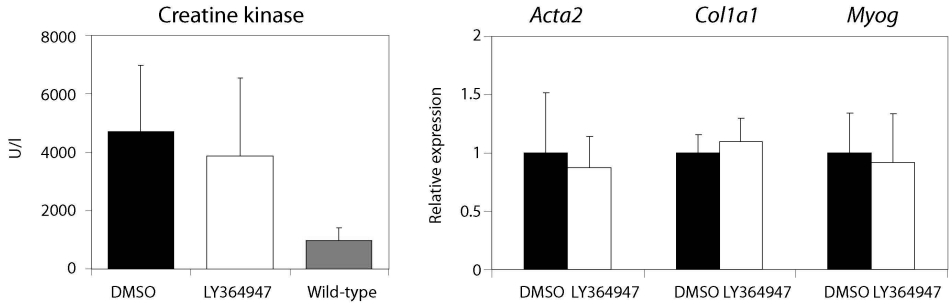


**Figure 5.3. Daily treatment of LY364947 did not alter dystrophic parameters in *mdx* mice.** LY364947 were administered via intraperitoneal injections into 5-6 weeks old *mdx* mice (n=3) at the dose of 1 mg/kg daily for 6 weeks. Serum was collected prior to sacrificing the animals for creatine kinase measurement. RNA was isolated from triceps and analyzed for fibrotic (*Acta2, Col1a1*) and regeneration (*Myog*) markers by QPCR, using *Gapdh* as housekeeping gene.

## 5.3. Evaluation and opportunities to develop TGF-β inhibitor in other muscle disease

Muscular dystrophies represent a large group of inherited disorders caused by mutations in genes encoding for several muscle proteins, including, but not limited to, the constituents of Dystrophin-associated glycoprotein complex (DGC). The clinical phenotypes and the primarily affected skeletal muscles differ but muscular dystrophy patients generally become susceptible to severe atrophy and show significant loss of muscle function. It is therefore interesting to speculate whether inhibition of TGF-β pathways can be beneficial for multiple muscle wasting diseases.

Muscle atrophy is an apparent phenotype of the mouse model of Limb-Girdle muscular dystrophy 1C (LGMD1C), which harbours a mutation in the *caveolin-3* gene. Transgenic crossing of caveolin-3 mutant with myostatin mutant mice, which harbours a frame-shift mutation leading to improper maturation of the protein (Nishi *et al.*, 2002), exhibit reduced atrophy and improved muscle strength (Ohsawa *et al.*, 2006). Interestingly, caveolin-3 interacts with ALK4 and ALK5 and inhibits their functions, suggesting that the pathology of LGMD1C is at least partly caused by hyperactivation of myostatin signaling. Correspondingly, administration of soluble ActRIIB-Fc ameliorates the atrophic phenotype observed in the *caveolin-3* mutant mice, suggesting its therapeutic potential for LGMD1C (Ohsawa *et al.*, 2006). Improved muscle force and mass was also observed upon AAV-mediated administration of myostatin propeptide in the LGMD2A mouse model, which is caused by mutation in *calpain 3* gene, but not in LGMD2D mouse model harbouring mutation in α-*sarcoglycan* gene (Bartoli *et al.*, 2007). In addition, in δ-*sarcoglycan* mutant mice, the mouse model for LGMD2F, administration of myostatin neutralizing antibody improved muscle mass and regeneration, and reduced fibrosis in the early stage, but was ineffective in the later stage of the disease (Parsons *et al.*, 2006). Furthermore, the loss of myostatin activity in the $dy^W/dy^W$ mouse model of laminin-deficient congenital muscular dystrophy, a much more severe and ultimately lethal disease model, does not improve any aspects of muscle pathology (Li *et al.*, 2005). Taken together, these studies suggest that partial myostatin inhibition may be beneficial to ameliorate several different types of muscular dystrophy, although the outcome of the intervention may differ due to disease severity.

Similarly, the benefit of myostatin inhibition cannot be generalized in the context of neurogenic muscle atrophy, such as in amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA). Myostatin neutralizing antibody did not delay the onset of the disease nor extend the survival in ALS mouse model (Holzbaur *et al.*, 2006). In SMA mouse model, either transgenic overexpression of follistatin or postnatal administration of ActRIIB-Fc did not extend the survival, despite a minimum increase in the muscle mass (Sumner *et al.*, 2009). However, follistatin gene transfer led to increased muscle mass, motor function and survival in SMA mouse model (Rose, Jr. *et al.*, 2009), but resulted in no survival extension in ALS (Miller *et al.*, 2006). The results obtained with different myostatin antagonists in ALS and SMA are inconsistent and therefore more research is needed. It is likely that the death of motor neuron cells is more aggravating to the disease pathology than any favourable contributions of non-neuronal cells can make up for, thereby myostatin inhibition may be ineffective to protect against the onset and progression of these diseases.

In addition to the inherited myopathies described above, the therapeutic potential of myostatin inhibition has also been investigated in several acquired myopathies, such as sarcopenia and cancer-associated cachexia. Sarcopenia is defined as the loss of skeletal muscle mass and function that occur with age. Neutralizing antibody and myostatin peptide have been shown to prevent the loss of body weight, muscle mass and function in aged mice (Siriett *et al.*, 2007; Murphy *et al.*, 2010). In the context of cancer-induced cachexia, implantation of Lewis lung carcinoma into myostatin mutant mice resulted in more severe loss of muscle mass than in wild-type mice (Benny Klimek *et al.*, 2010), suggesting that a lack of myostatin signaling from early stages of development might make muscle more susceptible to atrophy. However, administration of ActRIIB-Fc profoundly reversed the loss of skeletal muscle and prevented cardiac atrophy, leading to prolonged survival (Zhou *et al.*,2010). Although encouraging, further development of this compound as a new therapeutic strategy for reversing cachexia-induced muscle wasting might be delayed given the side effects observed in clinical trials with DMD patients.

Even though muscle wasting and, in general, a hostile milieu in muscle are observed in the inherited and acquired myopathies described above, the response to myostatin intervention differs between disease models. The outcome was sometimes inconsistent even within the same disease models, which might be caused by different disease stages at which the intervention was given.

Notably, AON-mediated exon skipping to correct the genetic damage has also been exploited in muscular dystrophies other than DMD, such as in dysferlinopathy (van Putten and Aartsma-Rus, 2011). Missense or nonsense mutations in the DYSF gene that lead to the absence of functional dysferlin protein result in LGMD2B (Liu *et al.*, 1998). AON-induced exon skipping is expected to restore dysferlin and its functionality, and proof-of-concepts have been achieved *in vitro* (Wein *et al.*, 2010; Aartsma-Rus *et al.*, 2010). Therefore, one might propose a combination of exon skipping to restore dysferlin restoration with inhibition of myostatin/TGF-β receptors (**Chapter 4**) to be beneficial for dysferlinopathy, and as such representing a very unique type of personalized medicine.

## 5.4. Comparison with other TGF-β inhibition strategies in non-muscle diseases

The TGF-β signaling pathway is essential for regulation of vasculogenesis and angiogenesis. Mutations in the genes for the TGF-β family components are therefore associated with several pathological conditions concerning vascular dysfunction, such as Marfan syndrome (MFS) and hereditary hemorrhagic telangiectasia (HHT), as well aggressive tumour angiogenesis.

Mutation in the *FBNI* or *TGFBRII* genes, which encode microfibrillar protein fibrillin, which controls TGF-β bioavailability or the type II receptor for TGF-β, causes MFS type I and II, respectively. MFS is characterized by defects in cardiovascular system including aorta and heart valves due to increased TGF-β signaling activity (Dietz, 2010). Inhibition of TGF-β signaling by TGF-β neutralizing antibody resulted in decreased aortic root dilatation, elastic fiber degeneration and Smad2 activation (Brooke *et al.*, 2008). Similar improvements were observed upon administration of losartan, an inhibitor of angiotensin II type I receptor (Habashi *et al.*, 2006; Cohn *et al.*, 2007). It has been suggested that losartan affects TGF-β signaling via crosstalk with the ERK pathway instead of the canonical Smad-mediated pathway (Holm *et al.*, 2011; Habashi *et al.*, 2011). Several multicenter clinical trials are ongoing to evaluate the benefit of losartan in MFS patients (Lacro *et al.*, 2007; Radonic *et al.*, 2010) and the results are eagerly awaited. Interestingly, losartan treatment also has beneficial impact on muscle architecture and function in *mdx* mice (Cohn *et al.*, 2007), as well as the cardiac and respiratory function (Bish *et al.*, 2011; Nelson *et al.*, 2011; Spurney *et al.*, 2011). It is not yet clear whether the reversal of skeletal myopathy achieved via losartan treatment is also mediated via ERK pathway as in the MFS mouse model.

TGF-β is also important for angiogenesis, which is an event of formation of new blood vessels by activation, proliferation, migration of endothelial cells from an existing blood vessel. The TGF-β signaling mechanism in the endothelial cells itself is unique, as it acts as a rheostat rather than an on-off switch. TGF-β can either inhibit endothelial cells migration and proliferation via the ALK5/Smad2/3 signaling pathway, or stimulate via the ALK1/endoglin signaling pathway leading to Smad1/5/8 activation (Goumans *et al.*, 2003; Lebrin *et al.*, 2004).

Several anti-tumor angiogenic therapies that interfere with TGF-β receptors predominantly expressed in endothelial cells, namely endoglin and ALK1, have been proposed (van Meeteren *et al.*, 2011). Several anti endoglin antibodies were reported to suppress tumour growth by selectively inhibiting the tumour-associated blood vessels, disrupting tumour-associated angiogenesis, as well as suppressing metastasis (Tsujie *et al.*, 2008; Uneda *et al.*, 2009). A clinical trial administering anti endoglin antibody in patients with advanced or metastatic solid cancer has recently been completed (NCT00582985). The treatment was well tolerated in all participants and, in some patients, decreased circulating tumour markers and prolonged stabilization of the disease. This finding is now being expanded and evaluated in patients with different solid tumour origins including prostate, bladed, ovarian and liver cancer (please refer to www.traconpharma.com for pipeline overview).

Next to endoglin, inhibiting angiogenesis by targeting ALK1 has also been explored. ALK1 is involved in endothelial cell migration by mediating not only TGF-β (Lebrin *et al.*, 2004), but also BMP-9 and BMP-10 signaling (Scharpfenecker *et al.*, 2007; David *et al.*, 2007). Using a soluble chimeric protein consisting of the extracellular part of ALK1 fused to an Fc-fragment (ALK1-Fc), Cunha *et al* showed reduced tumour growth and progression due to inhibition of

angiogenesis in pancreatic mouse model (Cunha *et al.*, 2010). Preliminary results of a clinical trial in multiple myeloma patients receiving ALK1-Fc (ACE-041, NTC00996957) showed that the treatment was well-tolerated and accompanied with >20% decline in tumour metabolic activity (www.acceleronpharma.com). Anti ALK1 antibody is currently also in clinical trial in patients with advanced solid tumours (NCT00557856), following encouraging inhibition of tumour angiogenesis in multiple tumour mouse models (Hu-Lowe *et al.*, 2011).

Despite the promising outcome so far, it is also important to see whether these strategies do not lead to inhibition of normal vasculogenesis in non-tumour tissues, because dysfunctional endoglin and ALK1 leads to fragile blood vessels (McAllister *et al.*, 1994; Johnson *et al.*, 1996). On the other hand, the predominant expression of endoglin and ALK1 in endothelial cells, as well as the unique balancing switch in this cell type becomes a major drive of exploration of these strategies for angiogenesis-related disorders.

The ALK1/endoglin arm of TGF-β signaling *per se* has not been directly associated with muscular dystrophy. Overexpression of endoglin in rat myoblasts decreases TGF-β-induced extracellular matrix synthesis, although the endogenous expression of endoglin in myoblasts itself is very low, if not absent (Obreo *et al.*, 2004). We also observed decreased TGF-β-induced luciferase upon downregulation of endoglin in fibroblasts (unpublished observation), suggesting an as yet unrevealed biological role of endoglin. Furthermore, we observed increased ALK1 expression in gastrocnemius muscle in several dystrophic mouse models, which correlate with increasing disease severity (**Figure 1.4**). However, it is not known yet which muscle resident cells express ALK1 and/or endoglin nor is it known if these receptors contribute to DMD pathology.

The contribution of different resident cells towards disease aetiology and how they response differently to TGF-β signaling is best reflected in chronic liver disease (CLD). Although the disease cause is not a mutation in any of the TGF-β signaling components, TGF-β is involved in and contributes to multiple stages in CLD. Initial liver injury will prompt regeneration. TGF-β induces apoptosis and oxidative stress in hepatocytes, thus inhibits liver regeneration. Furthermore, TGF-β represses inflammation responses by activating regulatory T-cells, as well as induces liver scarring via transformation of hepatic stellate cells to myofibroblasts, leading to development of cirrhosis, which is characterised by architectural disruption, extensive fibrosis, nodule formation and vascular changes. Cirrhotic livers have the tendency to develop towards malignant tumours, where TGF-β exacerbates by facilitating angiogenesis and inducing epithelial-mesenchymal transition (Dooley and ten Dijke, 2012).

Several anti TGF-β approaches have been successfully used for the treatment of carbon tetrachloride-induced fibrosis, such as overexpression of Smad7, a natural TGF-β inhibitors or administration of cytokines that induce Smad7 expression such as interferon-γ (Dooley *et al.*, 2003; Breitkopf *et al.*, 2005). However, in humans, the end phase can take several decades to be established, and inhibition of TGF-β will have either adverse or beneficial effects depending on the disease stage. Even though CLD is not progressing as rapidly as DMD, timing is important for intervention for both diseases. Furthermore, given the different cell-types that contribute to the disease aetiology of DMD and CLD, interference with cell-type specific signaling mechanism (**Chapter 2**) may be beneficial as therapeutic intervention.

## 5.5. Bringing DMD therapy to a reality

Drug development is a very costly and lengthy process. Only a few drugs will go into trials and even fewer will eventually get market approval. Thus, developing a therapy for DMD is a very long process. There are many therapeutic approaches that have entered clinical trials, yet many more are still in development, and new promising strategies are being proposed regularly. As outlined above, future therapies for DMD can be foreseen to be combinatorial approaches targeting the primary genetic defect and combating secondary symptoms. This section provides some evaluations and recommendations on how to move it forward.

In **chapters 3** and **4**, new exon skipping strategies targeting myostatin and myostatin/TGF-β receptors were introduced, with a view to combine it with the dystrophin exon skipping to provide a more "holistic" treatment regime. In **chapter 4**, it became evident that targeting the activity of multiple TGF-β ligands is more beneficial for the dystrophic phenotype than targeting a single ligand, thus exon skipping in ALK4 and ALK5 would hold greater promise than in myostatin alone.

An advantage of this combinatorial approach is the use of the same pharmacological class of compounds as the AON targeting *DMD*, thus in principle they can be combined in single administration. Importantly, we showed that several AONs in such a combined administration did not interfere with one another, although checking potential complementarities between two or more AONs is required (**Chapter 3**). As DMD AONs have been shown to be well tolerated in clinical trials, such chemical compounds might provide a safer option than for instance gene transfers. However, one should realize that AONs targeting two different genes are still considered to be different drugs, thus they will have to go through the same assessments as for other new drugs, including assessment of benefits and toxicities in large animals prior to human trials.

In addition to these exhaustive but necessary steps, one should realize that an AON that is effective *in vitro* is not always effective *in vivo* (**Chapter 3**). Since sequences necessary for testing in animal models may be different from the sequences to be used in humans, this confers a risk to clinical development programs. Furthermore, AONs would need to be delivered systemically to become a viable therapy, which might decrease the efficiency. Large amount of AONs will end up in liver and/or kidney instead of the targeted tissues, *e.g.* muscle (Heemskerk *et al.*, 2010). This may also lead to unwanted side effects in the non-targeted tissues. Such challenging aspects may be resolved in the future by the development of new chemistry modifications, such as muscle-targeting peptides that can be conjugated to an AON (Yin *et al.*, 2010b; Yin *et al.*, 2011a; Yin *et al.*, 2011b). These strategies will enhance the delivery and muscle tissue uptake but may come with potential toxicity and immunogenicity issues.

Despite these extensive challenges, the results presented in this thesis suggest a novel range of application areas for exon skipping AONs, both as therapeutic agents and research tools. The results reflect the versatility of the exon skipping technology and suggest that they become a powerful tool for customizable and individualized treatment strategies.

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