SUMMARY

Nonsense mutations in the gene encoding dystrophin cause Duchenne muscular dystrophy (DMD), a lethal and debilitating neuromuscular disorder. Dystrophin is an important muscle structural protein that protects muscle membrane from contraction-induced damage. Therefore, in the absence of dystrophin, the integrity of muscle fibers will be compromised and severe degeneration will take place. When the regeneration process mediated by satellite cells can no longer compensate, muscle fibers is eventually replaced by connective or fibrotic tissue, leading to the loss of muscle function.

Multiple stages in DMD pathology are associated with the Transforming Growth Factor (TGF)-β signaling pathway (**Chapter 1**). The TGF-β superfamily consists of more than 30 secreted proteins including TGF-β, bone morphogenetic protein (BMP), activin/inhibins and growth and differentiation factor (GDF). These proteins regulate many biological processes, such as cell growth and differentiation, and maintain homeostasis during development and in multiple adult tissues.

To elicit these diverse physiological responses, a fairly simple and yet powerful signaling pathway is utilized by the TGF-β family members. The basic signaling engine consists of two receptor serine/threonine kinases, termed receptor types I and II, and intracellular Smad proteins. The ligand assembles a receptor complex that activates Smad proteins, which will assemble multisubunit complexes that regulate transcription. Two general steps thus actually suffice to carry the TGF-β stimuli to target genes.

How can such a simple system mediate a variety of cell-specific gene response? It is now apparent that TGF-β signaling pathways have equally important extracellular and intracellular control mechanism. This includes myostatin (GDF-8), one of the members that is highly expressed in skeletal muscle. In addition to being a negative regulator of myoblast differentiation, myostatin also plays role in adipogenesis, skeletal muscle fibrosis and myometrial cell proliferation. Genetic mutation of myostatin leads to a remarkable increase of muscle mass, but, as myostatin is found in the circulation, effects on other tissues are somehow expected.

We hypothesized that such remarkable effects of myostatin in the muscle are controlled by a unique modulatory mechanism. Indeed, we found that myostatin signaling in myogenic and non myogenic cells are conferred by different utilization of type I receptors, which are also termed activin receptor-like kinases (ALKs), and co-receptor (**Chapter 2**). In myogenic cells, myostatin signaling is dependent on activin receptor-like kinase-4 (ALK4), whereas ALK5 is utilized in non myogenic cells. Furthermore, we found that the ALK4-dependent myostatin signaling in muscle is largely conferred by a membrane-associated co-receptor Cripto, which is predominantly expressed in myogenic cells but absent in non muscle cells. Moreover, Cripto has different influences on TGF-β family members that play a role in muscle, *i.e.* myostatin, activin and TGF-β. As such, Cripto may also be an interesting therapeutic target to follow up in the future.

As DMD is caused by the lack of dystrophin, one strategy is to bring back dystrophin in the dystrophic muscle. Antisense oligonucleotide (AON)-mediated exon skipping has been used to reframe the mutated *DMD* gene and restore dystrophin protein synthesis. It will, however, be less effective in the later stage of the disease where fibrosis is already extensive. This thesis explores the possibility of using exon skipping AONs to inhibit several components of the TGF-β family signaling and blunt their inhibitory effects on muscle regeneration and fibrosis.

In **Chapter 3**, we first used AONs to functionally knockdown myostatin expression. They efficiently downregulate myostatin *in vitro*, but induce only subtle exon skipping *in vivo*. Nevertheless, in a relatively straightforward manner, we were able to combine myostatin and dystrophin AONs and induce exon skipping of both genes without functional interference. This provides a conceptual foundation for a combinatorial therapeutic approach, which targets the primary genetic defect and attempts to improve muscle quality.

We further sought to use AON to functionally knockdown myostatin and/or TGF-β receptors ALK4 and/or ALK5 (**Chapter 4**). This strategy allowed us to target the activity of a broader spectrum of TGF-β members, including but not limited to myostatin. Administration in dystrophic mice reduces fibrosis in the diaphragm, which is known to be the most affected muscle. Interestingly, combination of both ALK4 and ALK5 inhibition induces most pronounced effects. The beneficial response after targeting ALK4 or ALK5 separately demonstrates the involvement of TGF-β and activin in DMD pathology. Overall, in addition to its therapeutic potential, the AON-mediated exon skipping approach also enables the dissection of the roles of TGF-β family members in muscle regeneration and fibrosis, and potentially other aspects of DMD pathology.

In summary, this thesis discusses how the inhibition of several members of the TGF-β signaling pathway has been implicated in ameliorating DMD pathology. Furthermore, it also increases the awareness that more knowledge on how these family members actually play role in (dystrophic) muscle may still be needed. Finally, alteration of TGF-β signaling components is involved in various diseases with multilayered pathophysiology, including but not limited to other neuromuscular disorders. Thus, the use of AONs has potential therapeutic value for other TGF-β-related disorders and is also important research tools to study the effect of modulation of TGF-β receptor family members in the different facets of these diseases.

SAMENVATTING

Nonsense mutaties in het gen dat codeert voor Dystrofine kunnen Duchenne spierdystrofie (DMD), een dodelijke en slopende neuromusculaire ziekte, veroorzaken. Dystrofine is een belangrijk structureel spiereiwit dat de spiermembraan beschermt tegen de schade die geinduceerd wordt door contractie van spieren. Bij de afwezigheid van het eiwit dystrofine wordt de integriteit van spiervezels aangetast. Wanneer het herstelmechanisme door satelliet cellen de achteruitgang van het spierweefsel niet langer kan compenseren, worden de spiervezels uiteindelijk vervangen door bindweefsel of fibrotisch weefsel en dit leidt tot verlies van spierfunctie.

Meerdere stadia van de DMD pathologie staan in verband met de Transforming Growth Factor (TGF)-β signaleringscascade (Hoofdstuk 1). De TGF-β superfamilie bestaat uit meer dan 30 uitgescheiden eiwitten zoals TGF-β, bone morphogenetic protein (BMP), activin/inhibins en growth and differentiation factor (GDF). Deze eiwitten reguleren veel verschillende biologische processen, zoals celgroei en differentiatie, en zijn belangrijk voor het behoud van homeostase gedurende de ontwikkeling en in verschillende volwassen weefsels.

Om deze verschillende fysiologische reacties te induceren gebruiken TGF-β eiwitten een vrij eenvoudige en toch krachtige signaleringscascade. Het basis signaleringscomplex bestaat uit twee receptor serine / threonine kinasen, receptor I en II genoemd, en intracellulaire Smad-eiwitten. Het ligand komt samen met een receptorcomplex dat op zijn beurt weer Smad-eiwitten activeert, die vervolgens weer samenvoegen tot multi-subunit complexen die de uiteindelijke transcriptie reguleren. Twee algemene stappen zijn dus eigenlijk voldoende om de TGF-β stimuli richting de genen te dirigeren.

Hoe is het mogelijk dat zo'n eenvoudig systeem een verscheidenheid aan cel specifieke genen tot expressie kan laten komen? Het is nu duidelijk dat de eiwitten uit de TGF-β familie zowel extracellulair als intracellulair diverse extra regelmechanismen gebruiken. Dit is bijvoorbeeld het geval voor myostatine (GDF-8), een van de leden die hoog tot expressie komt in spierweefsel. Naast het feit dat myostatine een negatieve regulator is van myoblast differentiatie, speelt het ook een rol in adipogenese, fibrose van de skeletspieren en myometriale celproliferatie. Genetische mutatie van myostatine leidt tot een aanzienlijke toename van de spiermassa, maar omdat myostatine in het bloed te vinden is, worden er ook effecten op andere weefsels verwacht.

We veronderstellen dat deze opmerkelijke effecten van myostatine in de spieren worden bestuurd door een uniek modulerend mechanisme. We hebben ontdekt dat myostatine signalering in myogene en niet myogene cellen wordt uitgevoerd door verschillend gebruik van type I receptoren, die ook worden aangeduid als activin receptor-like kinases (ALKs), en een co-receptor (hoofdstuk 2). In myogene cellen is myostatine signalering afhankelijk van activin receptor-like kinase-4 (ALK4), terwijl in niet myogene cellen ALK5 wordt gebruikt. Verder hebben we gevonden dat de myostatine signalering via ALK4 in spier grotendeels wordt aangestuurd door een membraan-geassocieerde co-receptor Cripto, die voornamelijk tot expressie komt in myogene cellen maar afwezig in andere celtypen. Bovendien heeft Cripto verschillende invloeden op de diverse TGF-β familieleden die een rol spelen in de spieren, zoals myostatine, activine en TGF-β. Toekomstige studies moeten uitwijzen of Cripto daarom ook een interessant therapeutisch aangrijpingspunt is.

Als DMD wordt veroorzaakt door het ontbreken van dystrofine, dan is een mogelijke strategie dystrofine in de dystrofische spier terug te brengen. Antisense oligonucleotide

(AON)-gemedieerde exon skipping is gebruikt om het gemuteerde DMD-gen te veranderen en de Dystrofine eiwit synthese te herstellen. Het zal echter minder effectief zijn in de latere fase van de ziekte waarbij fibrose al aanwezig is. Dit proefschrift onderzoekt de mogelijkheid van het gebruik van exon-skipping AONs die de verschillende onderdelen van de TGF-β signaleringscascade blokkeren en de stimulerende werking op fibrose en remmende werking op spier-regeneratie daarmee opheffen.

In hoofdstuk 3 hebben we voor het eerst AONs gebruikt voor een functionele knockdown van myostatine expressie. De AONs zorgen voor een efficiënte downregulatie van myostatine expressie *in vitro*, maar ze veroorzaken alleen subtiele exon-skipping *in vivo*. Op een relatief eenvoudige manier hebben we myostatine en dystrofine AONs gecombineerd en exon-skipping geïnduceerd in beide genen zonder functionele storing te veroorzaken. Dit zorgt voor een conceptuele basis voor een gecombineerde therapeutische aanpak, welke zich richt op het primaire genetisch defect en probeert de spierkwaliteit te verbeteren.

Verder hebben we gezocht naar AONs die zorgen voor een functionele knockdown van de myostatine en / of TGF-β receptoren ALK4 en / of ALK5 (hoofdstuk 4). Door toepassing van deze strategie wordt een breder scala aan TGF-β familieleden beïnvloed. De toediening van AONs in dystrofische muizen vermindert fibrose in het diafragma, waarvan bekend is dat deze spier zwaar aangedaan is. Het is interessant om te zien de combinatie van beide ALK4 en ALK5 AONs tot het meest uitgesproken effect leidt. De gunstige respons na de toepassing van AONs tegen ALK4 of ALK5 afzonderlijk toont de betrokkenheid van de TGF-β en activine bij DMD pathologie. In aanvulling op de therapeutische mogelijkheden, maakt de AON-mediated exon skipping aanpak het ook mogelijk om de rol van TGF-β familieleden in spier-regeneratie en fibrose en mogelijk andere aspecten van DMD pathologie te ontleden.

Kortom, in dit proefschrift wordt besproken hoe door middel van remming van de verschillende eiwitten van de TGF-β-signaleringscascade de progressie van DMD pathologie kan worden tegengegaan. We beseffen ook dat er nog steeds meer kennis nodig is over hoe deze familieleden een rol spelen in de (dystrofische) spieren. Tenslotte zijn diverse TGF-β signaleringscomponenten betrokken bij andere ziekten met complexe pathofysiologie, zowel neuromusculaire als andere stoornissen. Zo heeft het gebruik van AONs mogelijk therapeutische waarde voor andere TGF-β-gerelateerde aandoeningen en is tevens een belangrijk onderzoeksmiddel om het effect van de modulatie van de TGF-β receptor familie op de verschillende aspecten van de pathofysiologie te bestuderen.

CURRICULUM VITAE

Dwi Utami Kemaladewi was born in Surabaya, Indonesia, on April 24th, 1986. After finishing high school in 2003, she challenged herself to move abroad and enrolled in the International Bachelor program of Life Sciences at the Hogeschool van Arnhem en Nijmegen, Nijmegen, the Netherlands.

As part of this study, she did several research internships. Her first project was with dr. Guus Koch at the Central Veterinary Institute in Lelystad, where she learned how to rapidly detect different strains of Avian Influenza viruses using molecular techniques. After that, she spent nine months working with dr. Eric Soupene and dr. Frans Kuypers at the Children's Hospital and Research Center in Oakland, California. She studied the activity of Atp8a1, a novel subclass of ATPAse protein using yeast vesicles as model and showed that it was able to selectively regulate aminophospholipid movement in the membrane.

In October 2007, she moved back to the Netherlands and pursued her PhD in Leiden University Medical Centre (LUMC), where she was investigating the role of TGF-β/myostatin proteins in muscle and tweaking their signaling pathways to develop several therapeutic strategies for Duchenne muscular dystrophy. This work, which was done under the supervision of dr. Peter-Bram 't Hoen, dr. Willem Hoogaars, Prof. Gert-Jan van Ommen and Prof. Peter ten Dijke, is presented in this thesis.

During her PhD period, Dwi was actively involved in several PhD student bodies, including the Leiden Promovendi Overleg. She also enjoyed organizing scientific and social events for fellow PhD students. Her projects include the monthly meeting for LUMC's Division 5 PhD students, screening of the "PHD Movie" and 16th Medical Genetic Center workshop in Bruges, Belgium.

From June 2012, she continues her training as a postdoctoral fellow in dr. Ronald Cohn's laboratories at the Johns Hopkins School of Medicine, Baltimore and the Hospital for Sick Children, Toronto. Her current research focuses on the role of polyamine synthesis and androgen receptor signaling pathways in congenital muscular dystrophy.

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*) Equal contributions

LIST OF ABBREVIATIONS

2OMePS	2'-*O*-methyl phosphorothioate
AAV	Adeno-associated virus
ACTA2	α-smooth muscle actin
ALK	Activin-like kinase
ALS	Amyotrophic lateral sclerosis
Acvr/ActR	Activin receptor
AON	Antisense oligonucleotides
BMD	Becker muscular dystrophy
BMP	Bone Morphogenetic Protein
CFC1	Cryptic
CK	Creatine kinase
CLD	Chronic liver disease
Col	Collagen
COX	Cyclo-oxygenase
Ctgf	Connective tissue growth factor
CXMD	Canine X-linked muscular dystrophy
Db	Dystrobrevin
Dcn	Decorin
Dg	Dystroglycan
DGC	Dystrophin-associated Glycoprotein Complex
DMD	Duchenne muscular dystrophy
Dysf	Dysferlin
EDL	Extensor digitorum longus
EGF-CFC	Epidermal growth factor-like – Cripto-FRL1-cryptic
eMyHC	embryonic myosin heavy chain
FDA	Food and Drug Administration
FLRG	Follistatin-like related gene
FSHD	Facioscapulohumeral muscular dystrophy
GASP	Growth and differentiation factor-associated serum protein
GDF	Growth and differentiation factor
GOT	Glutamic-oxaloacetic transaminase
GPT	Glutamic-pyruvic transaminase
GRMD	Golden retriever muscular dystrophy
GS	Glycine/Serine
hDMD	humanized DMD mouse model
HHT	Hereditary hemorrhagic telangiectasia
Lam	Laminin
LGMD	Limb-Girdle muscular dystrophy
MFS	Marfan syndrome
MMP	Matrix metalloprotease
Mstn	Myostatin
NFKβ	Nuclear factor Kβ
nNOS	neuronal Nitric Oxide Synthase

PAI	Plasminogen activator inhibitor
PMO	Morpholino
SCID	Severe combined immunodeficiency
Sg	Sarcoglycan
SMA	Spinal muscular atrophy
SSPN	Sarcospan
Syn	Syntrophin
TAK	Tgf-β activated kinase
Tgfbr	TGF-β receptor
Tdgf	Teratocarcinoma-derived growth factor
TGF-β	Transforming Growth Factor-β
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor-α
Utrn	Utrophin
VM	Vivo morpholino

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Don't blame the boss, they have enough problems – Donald Rumsfeld

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Don't despise the empiric truth. Lots of things work in practice, for which the laboratory has never found proof – Martin Fischer

From den Dunnen lab, 1st of all, Johan, you are such a great, caring leader, always coming with challenging questions. Sandra, I learned a lot from you. Thanks for such a great help (zonder goede analist, ben je nergens ☺)! Maaike and Hans, I enjoyed every single second struggling yet having fun with you guys in the lab and "cow-office". Pietro, I'm so gonna miss you and all the chats we had (either important or not :p) every single day! Where else am I gonna find two lovely girls to share my passion of sweets, dresses, girly pajamas parties and who are not afraid of lab ghosts, like Eleonora and Irina? Thanks for all the supports esp. in the final year. I have met a lot of wise people along the way, including Marcel (appel en banaan-eten elke dag, heel gezond!), Isabella (for the chats in the cell lab, foreign postdocs tips, also for the closest-to-Italian cappuccinos), Ingrid (survival instinct after being trapped in Boston during hurricane Irene) and Christa (my 1st intramuscular guru!)! I worked here and there together with Silvana, Emile, Laura, Margriet, Nisha, Joyce, thank you very muchos! I have experienced very warm friendships from Yu, Stijn, Ivo, Yuching, Herman, Emmelien, and the Huntington's Willeke, Melvin (my cell lab wing-man!), Menno, Barry, Tassos and everyone in Lab-J. At some points I might have managed to bug everyone at the LGTC, esp. Henk, Yahya, Michel, Rolf, Yavuz, Sophie, for either equipment-related or just by crashing at their drinks ☺. Previous lab-J members: Eddy, Ashwin, Jacopo, Polina, Vishna, Matt, Judith, let's cross path again someday! My students Sem, Alex and Elena, it was great working with you all, all the best with your studies/works.

Just as bacterial cultures flourish in an agar-filled petri dish, laboratories are ideal breeding grounds for close friendships – Irene Levine

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Today's brightest researchers understand that science does not take place in a vacuum - Daniel Glaser

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Life might get busy but good friends are never forgotten - unknown

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We are the people our parents warned us about – Jimmy Buffet

Mama, makasih buat dukungannya ke adek. Maafin adek kalo ngga bisa temenin Mama tiap hari, jarang pulang, jarang telfon.. huhu.. Adek sayang Mama! Papa, meskipun Papa ngga ada disini, adek terus berusaha bikin Papa bangga. Adek sayang Papa, semoga Papa bahagia disana!

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❤ Dwi.