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Preclinical therapy development in FSHD: evaluation of pathophysiological aspects and therapeutic intervention in FSHD mouse models

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Appendix

English summary

Facioscapulohumeral muscular dystrophy (FSHD) is a progressive skeletal muscle disorder that mainly affects the muscles of the face, shoulders and upper arms. Skeletal muscle wasting in FSHD is caused by the failure to epigenetically repress the transcription factor DUX4 that is typically expressed during early development. DUX4 expression in skeletal muscle induces several myotoxic cascades that ultimately lead to the death of skeletal muscles cells. In most individuals with FSHD, the derepression of DUX4 is caused by a contraction of the DUX4 encoding D4Z4 repeat array to 1-10 units on chromosome 4q35 (FSHD1). A limited number of cases is caused by the inheritance of a relatively short D4Z4 repeat array (8-20 units) in combination with a mutation in one of the epigenetic repressors of the D4Z4 repeat array, specifically SMCHD1, DNMT3B and LRIF1. Currently, there is no therapy that can prevent or delay skeletal muscle wasting in individuals with FSHD.

Over the last few years several transgenic FSHD mouse models have been generated. These models can be used to study the pathological consequences of DUX4 activation in the skeletal muscles. In addition, these mice can be used to test new therapies. The main aim of this thesis was to characterize (and develop) new FSHD mouse models. Furthermore, we tested a novel RNA therapy that aimed to downregulate the DUX4 transcript in mice.

In **chapter 2** the role of Dnmt3b on the regulation of the D4Z4 repeat array in mice carrying a FSHD1-sized D4Z4 repeat array (D4Z4-2.5 mice) was studied. *DNMT3B* was previously identified as a FSHD2 gene in two families. In mouse embryonic stem cells (mESCs) derived from D4Z4-2.5 mice, murine Dnmt3b was able to bind to the human D4Z4 repeat array. Upon reducing Dnmt3b levels by siRNA transfection, DUX4 levels were increased in D4Z4-2.5 mESCs, confirming that Dnmt3b is involved in the repression of the D4Z4 repeat array. To study the role of Dnmt3b on *DUX4* repression *in vivo*, hemizygous D4Z4-2.5 mice were cross-bred with mice carrying a heterozygous hypomorphic *Dnmt3b* variant (Dnmt3b^{MommeD14} mice). The Dnmt3b^{MommeD14} variant did not enhance DUX4 expression and no phenotype was observed in the skeletal muscles of D4Z4-2.5 mice. Additionally, in most non-muscle tissues, DUX4 transcript levels were not changed in D4Z4-2.5/Dnmt3b^{MommeD14} mice compared to D4Z4-2.5 mice. In the inguinal lymph nodes and spleen, however, the Dnmt3b^{MommeD14} variant resulted in higher DUX4 and murine DUX4 target gene expression. Furthermore, although the Dnmt3b^{MommeD14} variant did not affect the chromatin compaction score of the D4Z4 repeat array, CpG methylation at the D4Z4 repeat array was slightly reduced. Taken together, our data shows that Dnmt3b has a modest role in the regulation of the D4Z4 repeat array in D4Z4-2.5 mice.

In **chapter 3** the skeletal muscle pathology of uninduced ACTA1-MCM;FLExDUX4 mice, a novel mouse model for FSHD, was characterized with focus on the inflammatory phenotype. Previously it was reported that compared to ACTA1-MCM;FLExDUX4 mice that are exposed to tamoxifen, DUX4 levels and target gene activation in uninduced mice are lower and the progression of the skeletal muscle phenotype is slower. We found that muscles of uninduced ACTA1-MCM;FLExDUX4 mice show several characteristics of skeletal muscle pathology, including smaller fiber sizes, myofibers with central nuclei, and macrophage infiltration. The

degree of skeletal muscle pathology increased with age. With RT-qPCR we measured a range of inflammatory markers in the skeletal muscles and found that several genes expressing complement factors, cytokines and chemokines were elevated compared to the levels observed in the skeletal muscles of ACTA1-MCM mice, with the most pronounced differences found when comparing older mice. Next, we used flow cytometry to determine which immune cells infiltrate the skeletal muscles. Most immune cells were $CD4^+$ T cells and pro-inflammatory macrophages, but other cell types like B cells, $CD8^+$ T cells and granulocytes were detected as well. Our work gives more insight into the skeletal muscle phenotype of uninduced ACTA1-MCM;FLExDUX4 mice and could be useful for other researchers who plan to perform studies with these mice.

In **chapter 4** a systemically delivered DUX4-targeting ASO was tested in uninduced ACTA1-MCM;FLExDUX4 mice. Generally, the treatment was well tolerated and no signs of severe organ toxicity were observed. In skeletal muscles of DUX4 ASO-treated mice the DUX4 transcript, DUX4 protein, and murine DUX4 target transcript levels were significantly reduced compared to control ASO-treated ACTA1-MCM;FLExDUX4 mice. Several markers for skeletal muscle pathology, including myofibers with central nuclei, collagen deposition, and macrophage infiltration, were reduced in DUX4 ASO-treated mice, suggesting that the DUX4 ASO can reduce the severity of the skeletal muscle pathology. Gene expression analysis in the quadriceps muscle next showed that the DUX4 ASO can reduce several pathways involved in inflammation, cell cycle, and fibrosis which are generally activated upon DUX4 expression. However, although DUX4 ASO-treated mice could run for a longer time period on a treadmill, most functional tests did not show an improvement and the skeletal muscle weight was not increased. Altogether, our study shows that the DUX4 ASO was effective in reducing DUX4 levels and the severity of skeletal muscle pathology, but needs further optimization to improve skeletal muscle function.

In **chapter 5** a new FSHD mouse model was developed that expresses constitutively high DUX4 transcript and protein levels in the skeletal muscles that can be used for therapy testing: the DUX4-F67A mouse model. As high DUX4 expression in skeletal muscles during early development is lethal, the *DUX4* transgene in this mouse model has a mutation in homeobox 1 that is required for DUX4 binding to DNA. The selected DUX4-F67A mouse line expresses high levels of the DUX4 transcript and DUX4 protein in the skeletal muscles, however murine DUX4 target genes are not activated. Although the skeletal muscles do not show a severe skeletal muscle phenotype compared to other FSHD mouse models, the expression of the disabled DUX4 protein resulted in atrophic muscles, a reduced muscle fiber size, a slight increase in fibrosis, and a change in fiber type composition. This suggests that DUX4 independent of its transcriptional activities can exert myotoxic effects in skeletal muscles. In addition, we also tested the DUX4 ASO in DUX4-F67A mice that was described in chapter 4. The DUX4 ASO reduced the DUX4 transcript by 75% and the DUX4 protein by 70% in several skeletal muscles. The DUX4 ASO treatment did not increase the weight of several skeletal muscles in F67A mice. In conclusion, because of the high DUX4 protein levels in skeletal muscles the DUX4-F67A mouse model is suitable to screen for DUX4-targeting therapeutics.

In addition, this model can be further used to study the role of DUX4 independent of its transcription activity.