Genetic causes and stem-cell-based therapeutic strategies in neuromuscular diseases

Chapter 8

Summary

Neuromuscular diseases are a broad group of heterogeneous disorders that impair normal function of neuromuscular systems, manifesting with a broad variety of symptoms. Muscle symptoms include muscular weakness, muscle wastage, muscular cramps, muscle spasticity, muscle pain, breathing and swallowing difficulties, with different onset and severity. Causes of neuromuscular disorders range from genetic mutations, injuries, viral infections, dietary deficiencies to certain drugs and poisons. Genetic neuromuscular diseases are usually complex and heterogeneous, which can be caused by a defect (or defects) in a single gene (e.g., myotonic dystrophy type 1, DM1), or in multiple genes (e.g., mitochondrial diseases) (**chapter 1**). Such highly clinical and genetic heterogeneity creates hurdles to identify the genetic defect for many neuromuscular disorders. However, whole exome sequencing (WES) brings the power to solve this issue, and enables identifying the underlying genetic cause and characterizing the unknown pathophysiological process. Despite the successes in identifying the genetic cause successful treatments are lagging behind. However, many novel gene and cell-based therapies are being developed these days and some have successfully reversed the phenotype in animal models of neuromuscular diseases (like CRISPR/Cas9 mediated correction in DM1 mice and mesoangioblasts infusion in dystrophic dogs). So promising treatments for these so far incurable neuromuscular diseases are emerging (**chapter 1**).

Our central hypothesis was that **(**1) whole exome sequencing followed by functional validation would enable us to identify the genetic cause of neuromuscular diseases and confirm the pathogenicity of the identified variants, especially in novel genes; (2) CRISPR/Cas9-mediated correction of known genetic defects in patient-derived muscle stem cells would be a potential autologous stem cell therapy for treating the muscle pathology.

Our first aim was to identify and functionally characterize gene defects in mitochondrial diseases. In **chapter 2**, we report a Dutch boy of non-consanguineous parents, with a clinical diagnosis of mitochondrial encephalomyopathy and complex I and IV deficiency, where whole exome sequencing revealed compound heterozygosity for two variants in the *SLIRP* gene, being a deletion in exon 3 and a splicing variant in intron 1. RT-PCR analysis revealed three *SLIRP* transcripts, adding up in total to only 63% of normal transcript levels in controls. The largest transcript, present in 18%, showed retention of a 106bp fragment of intron 1, leading to a frameshift and premature stop codon. The two smaller fragments contained either the deletion (74%) or wild-type *SLIRP* (8%). SRA

stem-loop-interacting RNA-binding protein (SLIRP) is predominantly localized in mitochondria and forms a complex with LRPPRC, which is involved in regulation of mitochondrial DNA (mtDNA) gene expression. Indeed, in fibroblasts of the patient a reduced RNA level of the mtDNA genes *MT-ND1*, *MT-ND6* and *MT-CO1* was observed. Lentiviral transduction of wild-type *SLIRP* cDNA in the patient fibroblasts increased the expression of the *MT-ND1*, *MT-ND6*, *MT-CO1* genes (2.5 to 7.2-fold), whereas transduction with the mutants did not. The decreased complex I and IV enzyme activities seemed to be the result of a reduced mitochondrial mass, reflected by a fourfold decrease of citrate synthase (CS) compared to total protein in patient fibroblasts. Transduction with wild-type *SLIRP* cDNA led to a 2.4-fold increase of CS compared to total protein and an increase in complex I and IV activities. The results of the complementation assay confirmed the causality of the *SLIRP* variants and revealed the mechanism, leading to the mitochondrial encephalomyopathy in this patient. In conclusion, we report the first variants in *SLIRP* gene as a novel cause of mitochondrial encephalomyopathy with combined complex I and IV deficiency.

Next in **chapter 3**, whole exome sequencing, analyzed with GENESIS and WeGET as 2 filtering strategies, revealed a homozygous deletion in the *C1QBP* gene in a patient with progressive external ophthalmoplegia (PEO) and multiple mtDNA deletions. The gene encodes the mitochondria-located complementary 1 Q subcomponent-binding protein, involved in mitochondrial homeostasis. Interestingly, of the reported eight patients with *C1QBP* variants, four were diagnosed with cardiomyopathy, but without involvement of the central nervous system. Our patient showed only isolated late-onset PEO without overt cardiac involvement. So, biallelic mutations in *C1QBP* cause mitochondrial cardiomyopathy and/or PEO with variable age of onset. Available data suggest that early-onset cardiomyopathy variants localize in important structural domains and PEO-only variants in the coiled-coil region. Anyhow, *C1QBP* mutations should be considered in PEO patients, regardless of cardiac phenotype.

Our second aim was to develop a generic autologous stem cell therapy to treat muscle pathology in neuromuscular disorders. Mesoangioblasts, which are myogenic stem cells, seem to be particularly suited for clinical application. Benefits of using mesoangioblasts as a somatic cell therapy product include abundantly availability from tissues, easy expansion to large numbers *in vitro* without losing proliferation potency, homing to skeletal muscle through the blood stream, allowing systemic delivery and efficient differentiation into skeletal muscle *in vivo*. Furthermore, mesoangioblasts were shown to ameliorate muscle function in

dystrophic dogs and mice.

In **chapter 4**, we analyzed mesoangioblasts from 30 clinically well-characterized DM1 patients. Recent studies demonstrated mitochondrial dysfunction in DM1 fibroblasts and peripheral blood mononuclear cells (PBMCs). However, little is known about the pathophysiology and mitochondrial function in myogenic progenitor cells of DM1 patients. Therefore, in this study, we characterized cellular hallmarks, mitochondrial content and metabolism in DM1 mesoangioblasts. Our results revealed that DM1 mesoangioblasts displayed typical DM1 hallmarks, including expanded CTG repeat and downstream splicing defects. The expanded CTG repeat size in DM1 mesoangioblasts positively correlated to the degree of muscle impairment while negatively to the age of onset. No clear correlation was observed between expanded CTG repeat size, mitochondrial content and mitochondrial respiration, in contrast, most DM1 mesoangioblasts displayed normal metabolism and mitochondrial function. Our results implicate that variation in cellular DM1 phenotype can exist among different cell types and addressed the importance of cell type specific research in DM1 pathology, thereby focusing on the relation of DM1 hallmarks and stemness of cells.

In **chapter 5**, we explored a novel CRISPR/Cas9-mediated genome editing approach in mesoangioblasts derived from six DM1 patients. Our approach was to abolish the splice-acceptor site of intron 14 in mesoangioblasts derived from DM1 patients, using CRISPR/Cas9-based Non-Homologous End Joining (NHEJ), in order to induce alternative splicing and exclude the CTG-repeat from the 3' UTR of the DMPK-RNA. We achieved a total editing efficiency of 36-51%, based on next-generation sequencing of the on-target site, and an increased expression of the alternative exon 16 downstream of the CTG repeats in six edited DM1 mesoangioblasts. In 9-27%, the expanded allele was corrected, which showed that our strategy was able to remove the expanded repeat from mesoangioblasts in sufficient numbers for eventual treatment, although it was still insufficient to see reversal of the cellular phenotype in the mosaic cell population. Currently, purification strategies are being explored to extract the correctly edited mesoangioblasts from the overall pool of edited and non-edited mesoangioblasts. Notably, next-generation sequencing of the ten most-likely off-target sites of the single guide RNA (sgRNA) revealed almost no mutations at those sites. This study demonstrates that CRISPR/Cas9 ribonucleoprotein-mediated genome edited DM1 mesoangioblasts provide a novel therapeutic strategy for DM1.

Chapter 6 reviews the state-of-art CRISPR/Cas9 technology, as it is one of the most important novel technologies in science, rewarded with the Nobel prize for its inventors Emmanuelle Charpentier and Jennifer A. Doudna in 2020. We give an overview of different CRISPR/Cas9 platforms, delivery formats and methods. We summarize the factors contributing to off-target events and approaches to avoid these undesired off-target events. We discuss the applications of CRISPR/Cas9 in different scientific fields, with a focus on therapeutic applications, as well as current ethic and regulation issues and a glimpse of future development.